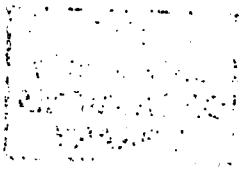

THE METABOLIC FATE OF DIETARY TERPENES IN FOLIVOROUS MARSUPIALS

by

Rebecca Reinga Boyle, B Pharm (Hons)

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Statement of Sources

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ABSTRACT

The metabolic fate of two commonly occurring dietary terpenes in Australian marsupial eucalypt folivores has been studied. The detoxification mechanisms employed by these folivores were considered from an ecological perspective. Interactions between herbivores and their respective diets has received considerable attention over the last quarter of a century. A popular hypothesis by Freeland and Janzen (1974) states that feeding behaviour of herbivores is influenced by limitations of the body's mechanisms for detoxifying plant secondary metabolites (PSM). However, very little is understood about the physiological processes and limitations of detoxification in wild animals. This study provides an insight into these processes in marsupial eucalypt folivores from a range of feeding niches.

An interspecies comparison was made of the metabolism of a bolus dose of the monoterpene *p*-cymene in a generalist herbivore, the brushtail possum (*Trichosurus vulpecula*), and three specialist folivores, the greater glider (*Petauroides volans*), the ringtail possum (*Pseudocheirus peregrinus*) and the koala (*Phascolarctos cinereus*), as well as in the laboratory rat (*Rattus norvegicus*) which provided a direct comparison to published data. Each animal was dosed, intragastrically, with single doses of *p*-cymene. Urine and faeces were collected pre- and post-dose.

Chronic ingestion of 1,8-cineole, emulating its natural occurrence in the diet of brushtail possums and koalas, was also studied. Possums were fed an artificial diet in which 1,8-cineole concentrations were manipulated to cover a range of concentrations while koalas were fed *Eucalyptus cephalocarpa*, which has a terpene profile dominated by 1,8-cineole.

Metabolites were identified by extraction, gas chromatography and mass spectrometry. Standards for many metabolites were isolated from urine to allow quantitation. A novel *p*-cymene metabolite, 2-*p*-carboxyphenylpropan-1,2-diol, was identified in the koala. Twelve novel 1,8-cineole metabolites were identified from brushtail possum and koala urine. Eleven of these were isomers of hydroxy cineolic acid. The isomeric structure and partial stereochemistry for 7-hydroxy-9-cineolic acid were determined by NMR and mass spectrometry.

Observational data from chronic ingestion of 1,8-cineole in possums demonstrated a threshold in maximum daily intake in most possums between 3 and 4 g/kg. The pattern of metabolites excreted in the brushtail possum during chronic ingestion demonstrated induction of enzymes responsible for producing the more oxidised metabolites during the first days of ingestion. There was no evidence of saturation of metabolic pathways associated with the larger intakes of 1,8-cineole.

For both terpenes, species-specific patterns of metabolite excretion were evident and reflected the natural occurrence of eucalypt leaves in the different diets. The number and degree of oxidation of metabolites, as well as the role of conjugation, varied depending on the natural feeding behaviour of the animals.

To facilitate studying the pattern of metabolism, terpene metabolites were grouped according to the total number of oxygen atoms (up to four) acquired during oxidation.

Overall there was a progressive increase in the proportion of more extensively oxidised metabolites; from the rat, to the generalist and through to the specialist folivores. The generalist utilised a multiplicity of non-specific oxidative pathways producing an array of metabolites covering all degrees of oxidation (9 metabolites of *p*-cymene and 18 metabolites of 1,8-cineole). On the other hand, the specialists had high capacity and specific oxidative pathways resulting in relatively few metabolites, all of which were radically oxidised.

Glucuronidation was important in the generalists, compensating for their inability to excrete the same degree of radically oxidised metabolites as specialists. Approximately 40 - 50 % of *p*-cymene metabolites and up to 60 % of 1,8-cineole metabolites were conjugated with glucuronic acid in brushtail possums. No significant conjugation of metabolites was observed in the specialists.

Increased polarity, whether achieved by glucuronidation or extensive oxidation, presumably results in the same overall enhanced capacity to excrete metabolites. It is proposed that, for specialists, oxidative efficiency reduces the necessity for subsequent conjugation, conserving glucuronic acid, a valuable resource in a nutritionally limited diet. The brushtail possum, however, consumes a varied and arguably better quality diet and can afford to excrete glucuronic acid in the detoxification of terpenes. Given that PSMs, such as terpenes, are always present in the eucalypt leaf diet of these folivores, these different strategies indicate that adaptation of detoxification mechanisms vary with dietary specialisation.

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GLOSSARY

Units and abbreviation

min	minute(s)
h	hour
ml	milliliter
l	litre
g	gram
kg	kilogram
µmol	micromole
mmol	millimole
nm	nanometer
atm	atomic mass units
°C	degrees celcius
rpm	revolutions per minute
psi	pounds per square inch
G	gauge
IU	international units
DM	dry mass
PSM	plant secondary metabolites
BMR	basal metabolic rate
CYP	cytochrome P450 enzymes
Cy	*p*-cymene metabolites
Ci	1,8-cineole metabolites

Chromatography abbreviations

GC	gas chromatograph or chromatography
FID	flame ionisation detector
MS	mass spectrometry
GC/MS	gas chromatograph/mass spectrometer or gas chromatograph/mass spectrometry
m/z	mass to charge ratio
EI	electron ionisation
CI	chemical ionisation
TMS	trimthylsilyl derivative
TIC	total ion current
SPME	solid phase microextraction
PDMS	polydimethylsiloxane
PFK	perfluorokerosene
HPLC	high performance liquid chromatograph or chromatography
pHPLC	preparative high performance liquid chromatography
LC/MS	liquid chromatography mass spectrometry
APCI	atmospheric pressure chemical ionisation
ESI	electrospray ionisation

MS/MS	tandem mass spectrometry
MW	molecular weight
M^+	molecular *m/z* ion
MH^+	protonated molecular *m/z* ion from positive ion MS
M^-	deprotonated molecular *m/z* ion
TLC	thin layer chromatography
pTLC	preparative thin layer chromatography
NMR	nuclear magnetic resonance
UV	ultraviolet

Statistical

sd	standard deviation
se	standard error
ANOVA, Anova	Analysis of variance
df	degrees of freedom
P	probability associated with a statistical analysis
CoV (%)	coefficient of variation (percent)
F	F statistic

Chemical names

NaOH	sodium hydroxide
$NaHCO_3$	sodium bicarbonate
HCl	hydrochloric acid
BSTFA	N,O-bis(trimethyl) trifluoracetamide

CHAPTER 1

PLANT-ANIMAL INTERACTIONS FROM THE PERSPECTIVE OF MARSUPIAL FOLIVORES AND *EUCALYPTUS* SPP.

1.1. Introduction

The acquisition of food is fundamental for the survival of all animals. For herbivores, however, the choice of diet is confounded by nutritional and toxicological parameters arising from a plant diet. As it is generally not in the interests of plants to be browsed upon, plants have evolved a battery of defensive mechanisms which protect them from herbivory. Adaptations can be physical (eg. prickles and spines) or chemical (plant secondary metabolites (PSMs); eg. terpenes, phenolics, tannins and alkaloids).

Furthermore, plants generally provide a poorer quality diet compared to an omnivorous or carnivorous diet (Cork and Foley 1991). However, the nutritional quality can be variable, depending on the plant species and parts ingested. Plant foliage, although abundant, provides the poorest quality of diet for herbivores. Fruit, seeds, flowers and shoots are generally less defended chemically as well as being of better nutritional quality (Cork and Foley 1991). Herbivores have necessarily evolved an array of adaptations to overcome specific plant defences, allowing them to extract sustainable nutritional benefit to subsist.

Additional constraints are imposed on herbivores occupying specific habitats and feeding niches (Cork and Foley 1991). Adaptive and optimal digestive strategies and nutritional requirements are important in mammals that are arboreal (ie. live in the forest canopy) as well as in mammals that are highly folivorous (ie. ingesting a foliage diet). Four Australian marsupials inhabiting temperate eucalypt forests are among the few mammals successfully occupying these niches. They are the common brushtail possum (*Trichosurus vulpecula*), common ringtail possum (*Pseudocheirus peregrinus*), greater glider (*Petauroides volans*) and koala (*Phascolarctos cinereus*).

Although foliage provides a potentially abundant food source, it is very fibrous and tough and often heavily defended with PSMs (eg. eucalypts). An important factor in the subsistence of folivores on such a poor diet is body size. Both allometric considerations and detoxification parameters contribute to the optimal body size. Allometry predicts that folivores should have large body masses. This is due to metabolic rate being proportional to (body mass)$^{-0.75}$. This means smaller mammals require more energy per unit weight (Nagy 1987; Freeland 1991). Furthermore, gut capacity is almost directly proportional to body size in all mammals (Parra 1978). Therefore for herbivores (and folivores in particular) to utilise a fibrous diet they need to either retain digesta for extended periods to allow adequate fermentation and digestion of cell walls, or have a high passage rate of digesta so sufficient energy can be extracted from the readily digested cell contents.

On the other hand, Freeland (1991) argued that PSMs are metabolised more rapidly in smaller animals. Scaling liver mass in relation to body mass, enzyme activity was

shown to increase with smaller body size, accounting for the increased capacity to metabolise PSMs. Freeland (1991) therefore suggests that dietary specialisation is more likely to occur in mammals with small body size.

Conveniently, folivores tend to fit into two size classes: very large (hundreds to thousands of kilograms, eg. elephants and giraffes) and moderately small (0.7 to 13 kg, eg. koalas, sloths and sportive lemurs; Cork and Foley 1991), providing support for both constraints on body size for folivores.

Body size is also a constraint of direct importance to arboreal mammals. To sustain mobility in the canopy, arboreal mammals require a small body size (less than 13 kg; Cork and Foley 1991). Metabolic and nutritional requirements of small herbivorous arboreal mammals can generally be satisfied if they utilise the best available diet (fruits, flowers, seeds and shoots). Strategies for overcoming the physical and physiological problems associated with being both arboreal and folivorous are considered further in this chapter, focusing on the arboreal eucalypt folivores of Australia.

One of the major issues in plant-herbivore interactions is the detoxification of ingested PSMs. The work presented in this thesis studied the metabolism and elimination of two monoterpenes in the plant-herbivore system of the *Eucalyptus* genus and the four arboreal marsupial eucalypt folivores. The aim of the study was to understand the metabolic fate of common eucalypt terpenes (*p*-cymene and 1,8-cineole; Figure 1.1) and to consider the metabolism strategies employed by each species from an ecological perspective. Elucidating species specific adaptations is fundamental to understanding the role of metabolism and detoxification in the ecology of these marsupials.

Freeland and Janzen (1974) argued 25 years ago that limitations and interspecific differences in enzyme detoxification would be important determinants of feeding niches of mammals. The hypothesis was based on observational studies correlating dietary PSM concentrations with diet selection. Since that time we have learned little about the actual physiological processes and limitations of detoxification in wild mammals. The hypothesis has never been challenged and there has been little data accumulated to refute or support it. Nonetheless, recent syntheses of mammal-plant interactions assume that detoxification processes and capacities are crucial to our understanding of current ecological patterns (eg. McArthur *et al.* 1991; Dearing and Cork 1999; Lawler *et al.* 1999).

The detoxification limitation hypothesis attempts to explain why browsing herbivores mostly consume varied diets (Freeland and Janzen 1974; Freeland and Winter 1975; Dearing and Cork 1999). A diet containing small amounts of a wide range of PSMs is argued to be less likely to saturate particular pathways of detoxification and excretion and so be preferable to a diet consisting of one plant type which would contain larger amounts of fewer PSMs. Nonetheless, specialist herbivores have minimal dietary variation and must therefore have high capacity detoxification pathways to deal with their PSM load. This study set out to test the hypothesis by comparing the metabolism of the eucalypt terpenes, *p*-cymene and 1,8-cineole, in generalist and specialist eucalypt folivores.

If terpenes are important in eucalypt-folivore interactions, then selective pressure on herbivores to evolve mechanisms to reduce toxic effects should be greatest in specialist herbivores and least in generalists, as this corresponds with the different levels of dietary terpenes normally consumed.

Detoxification strategies employed by eucalypt folivores to eliminate terpenes is only one issue in the complex interactions between these marsupials and their diet. It is becoming apparent that selection of preferred browse is multifactorial, balancing nutritional gain versus capacity for coping with both physical and physiological challenges. In order to put the significance of adaptive metabolic strategies into perspective an overview of the genus *Eucalyptus* and its defence mechanisms and of the eucalypt folivores and their known adaptations for subsisting on their respective diets follows.

1.2. Description of *Eucalyptus* spp

Eucalyptus is a large genus of evergreen trees that includes about 700 species. (Brooker and Kleinig 1990). Trees can range from small alpine shrubs to forest giants. Temperate forests in Australia are dominated by *Eucalyptus* spp. and ninety percent of the trees inhabited by arboreal marsupials are of this genus. In any location, usually five or fewer species represent 90 % of the trees present (Cork 1984). The leaves are protected with an indigestible waxy coating, are tough and fibrous and contain considerable amounts of PSMs (Cork 1984). Concentrations of lignin, tannins, phenolics and terpenoids are generally high, and can vary markedly between species and between individuals of the same species (Southwell 1973; Cork 1984). The variable, but characteristic, aromatic smell of eucalypt leaves is due to complex mixtures of terpenes (Boland and Brophy, 1991).

1.2.1. Nutritional quality of a *Eucalyptus* diet

The nutritional quality of eucalypt foliage may differ substantially between and within species as well as with the age of the leaf (Cork 1984). Furthermore, the ability of arboreal folivores to utilise the foliage may also differ significantly (Lawler *et al.* 1999; Cork 1984). A diet of eucalypt leaves is nutritionally poor, being low in protein, high in cell wall, or fibre, content and also it contains PSMs, which are potentially toxic and can reduce the digestibility of nutrients in the diet.

Most of the metabolisable energy obtained from eucalypt leaves comes from the cell contents which include simple carbohydrates, proteins and lipids (Cork *et al.* 1983; Foley *et al.* 1989). Eucalypt folivores obtain only 8 to 16 % of their daily digestible energy intake from fermentative digestion of cell wall polysaccharides (Cork and Foley 1991). Digestion of cell wall carbohydrates, such as cellulose, by microbial enzymes is inhibited by lignin, necessitating long fermentation times.

The majority of nitrogen is in the form of free amino acids and the overall content is relatively low, less than 2 % dry matter (Cork 1984; Degabriele 1981). Leaf tannins can potentially form complexes with dietary amino acids upon cell disruption,

making the amount of available nitrogen variable and low (McArthur *et al.* 1991; McArthur and Sanson 1993).

In species with high concentrations of terpenes, there is a poor conversion from digestible energy measurements to actual metabolisable energy values of the leaf. The physicochemical properties of terpenes (which are small lipophilic hydrocarbons) account for the high digestible energy values, whereas in fact these compounds are non-nutrients and typically require metabolism to enable their excretion from the body (Southwell 1978).

Young leaf is preferred by some marsupial folivores (eg. the greater glider and ringtail possum) and is generally higher in nitrogen and lower in cell wall content compared to mature foliage, although lignin and phenolics can be higher in the young leaves. However, the differences between leaves of different ages are not always great (Cork 1984).

A large number of studies have attempted to correlate nutritional and toxic parameters of eucalypt leaf diets such as nitrogen, fibre, water, tannin and volatile concentration with selection or rejection of leaf by marsupial eucalypt folivores (Eberhard *et al.* 1975; Ullrey *et al.* 1981; Cork *et al.* 1983; Hindell and Lee 1990; Hume and Esson 1993;(Lawler *et al.* 1998) Lawler *et al.* 1998). There has been no consistent correlation between any single factor and preferred browse. It is therefore becoming clear that leaf selection is based upon a balance of all these parameters, with each species selecting the best available diet that will most adequately meet its energy requirements.

1.2.2. *Eucalyptus* physical defences

1.2.2.1. Lignin

Generally the concentration of lignin in the cell walls of tree foliage, including eucalypt foliage, is high and the ratio of lignin to other cell wall constituents is the highest of all plant tissue (Ullrey *et al.* 1981; VanSoest 1982; Cork 1984). Lignin is generally resistant to any form of degradation in the gastrointestinal tract and can therefore be considered a physical defence against herbivory as it renders eucalypt leaf tough and fibrous (vanSoest 1982). As mentioned in the previous section lignin inhibits microbial enzymes, reducing the digestion of cell wall carbohydrates such as cellulose.

1.2.3. *Eucalyptus* chemical defences

1.2.3.1. Terpenes

Terpene profiles indicate eucalypts contain similar types of terpenes, although qualitative and quantitative amounts of individual terpenes and overall levels vary (Southwell 1973; Eberhard *et al.* 1975; Morrow and Fox 1980; Lawler and Foley 1999). Intra- and inter-species variation in the terpene composition is also highly variable (Southwell 1973; Southwell 1978; Foley *et al.* 1987; Li 1993).

Terpenes are the main PSM component of *Eucalyptus* oils and are recognised as multiples of the 5 carbon isoprene units (C_5H_8; Loomis and Croteau 1973; Boland and Brophy 1991). Monoterpenes are synthesised from two isoprene units to form 10 carbon skeletons while sesquiterpenes are comprised of three isoprene units resulting in 15 carbon skeletons. They are biosynthesised from mevalonic acid in a large number of plant genera (Li 1993) and sequestered in vacuoles within the leaf, thus protecting the cellular metabolic processes of the leaf from toxicity (Boland and Brophy 1991; Gershenzon 1994; Lawler and Foley 1999).

Terpenes may be classified as either acyclic, monocyclic or bicyclic compounds, and sesquiterpenes may also form tricyclic structures. Further functionalisation of these basic structures results in a large number of possible structures of both mono- and sesquiterpenes. Terpenes occur in six of the seven subgenera of *Eucalyptus* and have been described in many of these species (Boland and Brophy 1991; Li 1993). As already mentioned, the quantity and profile of terpenes can vary between species and even between individual trees (Southwell 1973; Southwell 1978; Nerg *et al.* 1994). However, there are a few monoterpenes that commonly occur in many eucalypt species and these include 1,8-cineole, *p*-cymene, α- and β-pinene, α-phellandrene and terpinene (Boland and Brophy 1991; Li 1993).

Monoterpenes appear to have little direct role in the growth or development of plants and therefore it has been suggested that their role is entirely ecological (Gershenzon and Croteau 1991). In the case of eucalypt leaves, their role appears to be to deter feeding by both insects and mammals. However, as mentioned previously, there is no consistent evidence of a correlation between monoterpene content of eucalypt leaf and leaf preference by marsupial folivores (Southwell 1978; Hume and Esson 1993). Therefore, although terpenes are potentially toxic, detoxification mechanisms have evolved allowing these species to ingest naturally occurring quantities of terpenes. However their detoxification, involving both oxidative modification and conjugation, has a metabolic cost which must be extracted from a poor quality diet (Cork and Sanson 1991; Foley and McArthur 1994). The energetic costs of oxidation and conjugation of PSM metabolites have not been investigated. However, the percentage of the total energy absorbed from the diet excreted directly as glucuronic acid has been calculated to be only 1 - 2 % in the koala. This represents, at most, 8 % of the glucose absorbed as dietary sugars and starches (Cork *et al.* 1983; Cork and Sanson 1991). These calculations do not take into account the metabolic costs of oxidation and conjugation reactions. In high oil yield eucalypt species, the cost is reflected in the disparity between digestible and metabolisable energy (Foley 1987).

Recently it has been proposed that a common eucalypt terpene, 1,8-cineole, acts as a marker for another group of potent anti-feedant compounds, diformylphloroglucinols (DFPGs; Lawler *et al.* 1998). DFPGs are relatively large molecules occuring in eucalypts which combine phloroglucinol with terpenes to form complex adducts (Lawler *et al.* 1998). It is proposed that DFPGs may not be readily detected by folivores until post-ingestive emesis occurs. In this study, the authors demonstrated that food intake correlated closely to the dietary concentration of DFPGs, and that terpenes such as 1,8-cineole may cue animals to the concentration of the DFPG toxins in the leaves (Lawler *et al.* 1999).

The two terpenes investigated in this thesis are *p*-cymene and 1,8-cineole and both commonly occur in eucalypts associated with marsupial folivores (eg. *E. viminalis, E. globulus, E. obliqua* and *E. radiata*; Bergin 1978; Boland and Brophy 1991). *p*-Cymene (4-isopropyltoluene) has a simple chemical structure based on an aromatic ring while 1,8-cineole (1,3,3-trimethyl-2-oxabicyclo[2.2.2]octane) is a monocyclic ether. Structures for *p*-cymene and 1,8-cineole are shown in Figure 1.1.

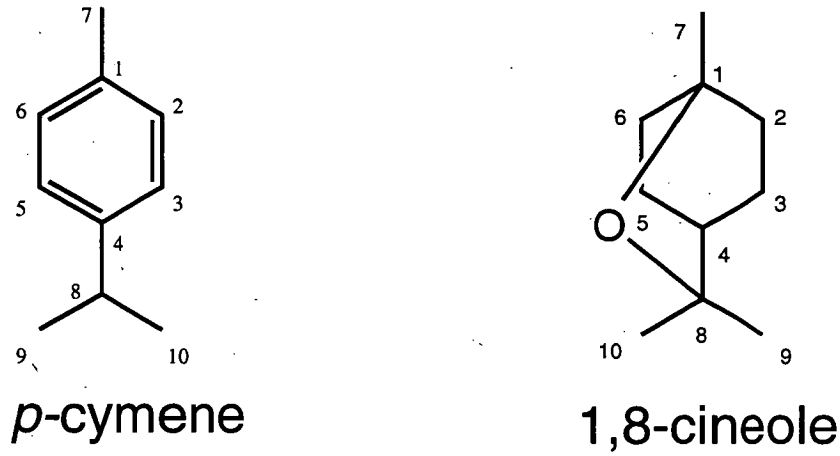


Figure 1.1. Chemical structures of *p* -cymene and 1,8-cineole, the terpenes investigated in this thesis.

1.2.3.2. Tannins

Tannin composition of eucalypt leaf is both qualitatively and quantitatively variable between species (Hillis 1966; Fox and Macauley 1977). Tannins are divided into two classes, condensed and hydrolysable. Hydrolysable tannins are characterised by a central glucose unit attached by ester linkages to several gallic acid groups or groups built up of gallic acid (Scheline 1991). They are readily hydrolysed by acid, base or enzymes to smaller phenolic compounds which are readily absorbed into the body and therefore require detoxification (McArthur *et al.* 1991). Condensed tannins are large polymeric compounds having molecular weights up to 20 000 (Harborne 1991). They are usually chemically stable under normal gastric conditions and therefore they are not absorbed into the body (Hagerman *et al.* 1992).

Tannins have an astringent taste and bind to protein (Harborne 1991). Therefore many affect diet selection, food intake and digestion while others cause direct toxicity to animals and require detoxification (Cork and Foley 1991).

1.3. Description of marsupial eucalypt folivores

It has been estimated that only 4 % of all mammalian genera contain species that are to some degree both arboreal and herbivorous (Eisenberg 1978). A number of Australian marsupials are among the most folivorous arboreal mammals. They fill these niches in both temperate eucalypt and tropical wet forests (Cork and Foley 1991). A brief description of each of the marsupial eucalypt folivores used in this study follows.

1.3.1. The common brushtail possum (*Trichosurus vulpecula*)

This species is highly variable in size and colouration. As with most marsupial species, animal size depends on the latitudinal location of a population; the largest animals occur in Tasmania where they commonly weigh up to 5 kg (personal observations) and the smallest animals occur in north Queensland (2 kg; Howe and

Kerle 1995). The brushtail possum has one of the widest distributions of any possum and is common throughout most of its range (Flannery 1994). Its range of habitation spreads from Cape York to south eastern South Australia and Tasmania. It also populates wooded areas in central Australia and south west Western Australia It has readily adapted to urban and rural habitats and may occur in high numbers in these areas. It was introduced into New Zealand in early to mid nineteenth century for the fur trade and has adapted well to the environment, creating major environmental problems for that country (Howe and Kerle 1995).

The brushtail possum prefers to den in tree hollows, however, it will also use burrows, fallen logs and man made structures (Flannery 1994). The brushtail possum is nocturnal, emerging at twilight. Females dominate the population (60 - 70 %) and the majority produce a single pouch young in the main autumn breeding season (Howe and Kerle 1995).

The brushtail possum consumes a varied diet. Plant matter forms the great bulk of the diet and includes foliage, fruit, buds and bark as well as pasture plants such as clover (Freeland and Winter 1975). *Eucalyptus* foliage forms part of the diet, but brushtails cannot be maintained on a single eucalypt species (Freeland and Janzen 1974; Dearing and Cork 1999). Insects and meat are also ingested at times of food shortage (Howe and Kerle 1995). Thus, the brushtail possum is a typical generalist herbivore (Dunnet *et al.* 1973; Kerle 1984; MacLennan 1984; Statham 1984).

1.3.2. The common ringtail possum (*Pseudocheirus peregrinus*)

This species is found throughout eastern Australia, from Cape York to Tasmania, and with an isolated population in the southwest of Western Australia (Flannery 1994). It inhabits a variety of vegetation types, requiring an understorey that forms a dense tangle of foliage. It commonly builds ball shaped nests, called dreys, which are constructed from twigs, leaves and shredded bark. Individual possums or family groups may have up to five dreys in their home range, often in close proximity

Figure 1.2. The four marsupial eucalypt folivores are shown in A) brushtail possum *(Trichosurus vulpecula)* , B) ringtail possum *(Pseudocheirus peregrinus)* , C) greater glider *(Petauroides volans)* and D) koala *(Phascolarctos cinereus).*

(McKay and Ong 1995). Ringtail possums are nocturnal, emerging from their dreys at twilight and being most active before midnight. They most commonly produce litters of twins, and when conditions are favourable, two litters per year.

The common ringtail possum is the smallest arboreal folivore to include *Eucalyptus* leaves as an important component of its diet. Indeed, this species occurs at the lower theoretical limit of body size for herbivores when allometric constraints are considered (ie. high metabolic energy requirements (Nagy 1987; Freeland 1991) and small absolute gut volume (VanSoest 1982)). Ringtail possums weigh between 700 and 1100 g, depending on the latitudinal location of the population (Flannery 1994).

Although *Eucalyptus* foliage is the preferred food item of the common ringtail possum, it is not eaten exclusively (Chilcott and Hume 1984). Foliage from other species such as *Kunzea* spp., *Leptospernum* spp. and *Acacia dealbata* has been reported in the stomach contents of ringtails, as well as flowers and fruit (Thomson and Owen 1964; Pahl 1984; Pahl 1987). The young expanding foliage of *Eucalyptus* species is the most preferred eucalypt foliage, with large, broad sapling leaves the next most preferred type, yet it can consume up to 50 % of its diet as mature leaves in most parts of its range (Pahl 1984; Pahl 1987). Unlike the brushtail possums, ringtails can be sustained successfully on a single species of *Eucalyptus* as their sole diet (Chilcott and Hume 1984).

Ringtails have an enlarged caecum, positioned at the top of the proximal colon, which retains and ferments fluid and fine particles from the digesta (Hume 1982). Ringtail possums are coprophagic. The contents of the caecum are evacuated and ingested as soft caecotrophs throughout the day, while hard faecal pellets are excreted throughout the night. This practice allows the salvage of nitrogen, in the form of microbial proteins, and energy, thereby reducing the ringtail possum's nitrogen requirements (Chilcott and Hume 1985). The species is a specialised leaf eater, but is not as selective for *Eucalyptus* spp. as the greater glider or koala.

1.3.3. The greater glider (*Petauroides volans*)

This species is the largest of the gliding marsupials, and can cover horizontal distances up to 100 m as well as change direction mid-flight. It differs from other gliding marsupials in that its gliding membrane reaches only to the elbow, and not the wrist. Its habitat is restricted to *Eucalyptus* forests in eastern Australia from Victoria in the south to the Barron River in northern Queensland. Its weight ranges from approximately 900 g in its northern extreme to 1700 g at its southern extreme (McKay 1995). It is strictly nocturnal animal and is solitary. It spends the days in tree hollows high in the forest canopy and produces a single offspring once a year (Flannery 1994).

It inhabits a variety of eucalypt forest types from low open coastal forests to tall forests in the ranges as well as low woodlands west of the Dividing Range (McKay 1995). It is distributed patchily throughout forest, with a large percent of a forest's population occurring in a small area (Braithwaite *et al.* 1983). The greater glider feeds only on one or two species of eucalypt at any one time and is similar to the koala in this regard. It is exclusively folivorous and is highly selective of young eucalypt foliage (Marples 1973; Kavanagh 1984; Foley 1987). It also has a large caecum,

which retains the fluid and fine particles of the digesta for microbial fermentation of cell wall components.

1.3.4. The koala (*Phascolarctos cinereus*)

This is the largest of the marsupial folivores with the average weight of north Queensland koalas being 5.1 and 6.5 kg for females and males, respectively and 7.9 and 11.8 kg respectively for Victorian females and males, respectively (Martin and Handasyde 1995). The koala is a sedentary animal, being inactive 20 h per day. Being sedentary is an advantage as it reduces energy requirements and therefore food intake (Martin and Handasyde 1995). Unlike most other arboreal mammals, the koala has no functional tail and relies upon its long limbs and large strong hands and feet for climbing. Koala habitat has been continually eroded by human activity and their range is now restricted to a patchy distribution from south eastern South Australia through to southern Victoria and to the Atherton Tablelands in north Queensland (Martin and Handasyde 1995).

Like the ringtail possum and greater glider, the koala has an enlarged caecum, which assists the fermentation and digestion of a highly fibrous diet. Although not coprophagic, young koalas ingest soft faecal pellets, called pap, excreted by their mother around the time of weaning. Apart from its nutritive value, this inoculates the alimentary tract with symbiotic microorganisms (Hume 1982; Osawa *et al.* 1993).

Of 700 species of *Eucalyptus,* koalas select their diet from approximately 35 species (Bergin 1978). Individual koalas therefore consume eucalypt leaves from only a few species in their home range. The leaf composition can be entirely made up of mature leaves when young foliage is scarce or inaccessible (Eberhard 1978; Lee and Martin 1988; Hindell and Lee 1990). Koalas carefully smell leaf before deciding whether to accept or reject it (Hume and Esson 1993).

1.4. Adaptations of marsupial eucalypt folivores

Marsupial eucalypt folivores have evolved the capability of utilising foliage and overcoming substantial physical and physiological problems. A brief discussion of some of the adaptations follows.

1.4.1. Physiological

1.4.1.1. Metabolism of absorbed PSMs

Specific metabolic detoxification strategies of leaf toxins in arboreal folivorous mammals have received little attention in the understanding of interactions between folivores and their leaf diet, and in the role of absorbed non-nutrients and toxins in diet selection. Although many biologically active compounds are detoxified as a consequence of metabolism, this is not always the case. For example, a monoterpene, pulegone, forms reactive metabolites which have been shown to be responsible for severe hepatotoxicity in mice, rats and humans (Thomassen *et al.* 1991; Nelson *et al.*

1992). For this reason, we cannot be sure that metabolism of PSMs equates to detoxification in every circumstance. The metabolism of absorbed terpenes and phenolics utilises standard biotransformation processes used in the elimination of endogenous and exogenous compounds. Enzymatic biotransformations result in metabolites which can be eliminated via either renal or biliary excretion. However, since there has been no consistent correlation found between diet selection and leaf toxins, the marsupial eucalypt folivores have apparently developed strategies which allow metabolism and elimination to occur at a rate which prevents the accumulation of these compounds to toxic levels.

1.4.1.1.1. Metabolism of terpenes

Metabolism of terpenes has been investigated in a number of species and has primarily been concerned with the safety of essential oils after human consumption when used for flavouring and fragrance, as well as in occupational health and safety of exposed workers. Metabolism of many terpenes has been reported in eutherian mammals, particularly rats, guinea pigs, rabbits and sheep (Ishida *et al.* 1979; Ishida *et al.* 1981; Walde *et al.* 1983; Asakawa *et al.* 1986; Madyastha and Srivatsan 1987; Asakawa *et al.* 1988; Madyastha and Srivatsan 1988; Ishida *et al.* 1989; Matsumoto *et al.* 1992) and has been reviewed by Scheline (1991).

Specific metabolism studies in marsupial eucalypt folivores are limited to just a few and were recently reviewed by McLean and Foley (1997). The earliest qualitative studies examined the metabolic fate of terpenes in koalas fed *E. punctata* (Southwell 1974; Southwell 1975). Metabolites of 1,8-cineole, limonene, *p*-cymene and α-phellandrene were shown to be products of firstly oxidation and then in some cases secondary conjugation with glucuronic acid or glycine. Hydroxy carboxylic acids and novel monoterpene lactones were considered to result from oxidative metabolism of α- or β-pinene and were excreted unchanged, or as labile glucuronides.

The metabolism of 1,8-cineole has been investigated extensively in the brushtail possum and has been shown to undergo multiple oxidations resulting in a complex pattern of metabolism (Flynn and Southwell 1979; Carman and Fletcher 1983; Carman and Fletcher 1984; Carman and Fletcher 1986; Carman *et al.* 1986; Carman and Klika 1991; Carman and Klika 1992; Bull *et al.* 1993; Carman *et al.* 1994; Carman and Rayner 1994). Investigations into the metabolism of α- and β-pinene, *p*-cymene and 1,8-cineole have also been reported in the brushtail possum, but were not exhaustive (Southwell *et al.* 1980).

Finally, the metabolic fate of terpenes has been investigated in ringtail possums fed *E. radiata* (McLean *et al.* 1993). The major terpenes in *E. radiata* included α- and β-phellandrene, *cis*- and *trans*-*p*-menth-2-en-1-ol and *cis*- and *trans*-piperitol. Mostly hydroxy carboxylic acids and dicarboxylic acids were found, plus two lactones that corresponded to those reported by Southwell (1974).

Both McLean *et al.* (1983) and Southwell (1974) suggested increased degrees of oxidation of terpene metabolites in marsupials specialising on *Eucalyptus* leaf. However, there is a paucity of detailed quantitative or comparative studies on terpene metabolite abundances in marsupials, and for that matter, in any species which encounters these compounds in their natural diet. The work presented in this

thesis addresses this deficit by providing a detailed quantitative study on the fate of two major eucalypt terpenes, *p*-cymene and 1,8-cineole, in marsupial eucalypt folivores.

Detoxification reactions and pathways are discussed in detail later in this chapter (see Section 1.5.3).

1.4.1.1.2. Metabolism of phenolics

Many non-tannin phenols, hydrolysable tannins, and even some condensed tannins which can be broken down in the digestive tract liberating smaller phenolic compounds, can be absorbed from the stomach and small intestine. These compounds must also be detoxified by conjugation reactions, possibly following further oxidation, and excreted via the kidney and bile (Freeland and Janzen 1974; Baudinette *et al.* 1980). The metabolism of phenol has been studied in a number of marsupials including the brushtail possum and koala. In both species, phenol was recovered almost exclusively as the glucuronide of phenol plus minor amounts of the quinol glucuronide (Baudinette *et al.* 1980).

1.4.1.2. Metabolic rate

Arboreal mammals generally have lower basal metabolic rates (BMR), or resting energy requirements, than the average for similar sized mammals (Nagy 1987). Folivorous arboreal mammals tend to have an even lower BMR than non-folivorous arboreal animals, and marsupials in general have lower metabolic rates than eutherians (Nagy and Martin 1985; Nagy 1987). The koala, along with the three-toed sloth, is the most folivorous mammal consuming mature leaves and has a BMR of 36 % of that predicted for a eutherian of the same weight (Nagy and Martin 1985). A low BMR has two advantages for marsupial folivores. First, the nutritional requirements are reduced, which, considering the nutritional quality of the diet, effectively reduces the amount of food required. Second, overall ingestion of PSMs is also reduced as a direct consequence of reduced food intake.

1.4.1.3. Nitrogen requirements

Marsupial eucalypt folivores have variable, species-dependent mechanisms for minimising losses, or improving efficiency of use, of dietary nitrogen, which allow them to extract sufficient nitrogen from their low nitrogen diet. A low BMR reduces the nitrogen requirements in all marsupials. However, high tannin concentrations and the fibrous nature of the leaf diet results in increased faecal losses of nitrogen. Tannin-protein complexes formed from either proline rich salivary proteins (discussed in the following section) or dietary proteins are at least partially hydrolysed in the koala caecum by enterobacteria (Osawa *et al.* 1993; Osawa *et al.* 1993). It is yet to be established whether similar enterobacteria occur in the other marsupial folivores. Furthermore, the low metabolic rate of koalas means that relatively small amounts of nitrogen are excreted as waste products (ie. urea and ammonia, Cork 1986). Ringtails reduce their nitrogen requirements by the production and ingestion of specially formed caecotrophs which contain microbial amino acids (Chilcott and Hume 1984; Chilcott and Hume 1985). Greater gliders ingest young foliage, which is generally higher in nitrogen compared to mature leaves (Cork and

Pahl 1984). Brushtail possums are able to feed on alternative food sources, presumably selected for their higher nitrogen content (MacLennan 1984).

1.4.1.4. Proline rich salivary proteins

High tannin diets result in elevated levels of faecal nitrogen, and this has been detected in eucalypt folivores (Cork 1986; Foley and Hume 1987; Foley and Hume 1987). Tannins bind with protein during digestion, forming complexes which reduce the digestibility of dietary proteins. However, in many herbivores, including the eucalypt folivores, the majority of faecal nitrogen loss has an endogenous source. Tannins have a very high affinity for proline rich salivary proteins, and these proteins have been found in a number of marsupials (eg. swamp wallabies, brushtail possums and koalas; McArthur *et al.* 1995). It is unclear whether the primary role of salivary proteins is to bind dietary tannins, however it has been shown in some eutherian species at least that the competitive binding between proline and dietary proteins with tannins allows greater absorption of dietary proteins and essential amino acids (McArthur and Sanson 1993; McArthur *et al.* 1995).

1.4.2. Anatomical

1.4.2.1. Gut

In most mammals that utilise plant tissues as food, there is some enlargement and specialisation of the gut to allow microbial fermentation of digesta and to increase the surface area available for absorption of digestion products (Hume 1982; Cork and Foley 1991). The marsupial eucalypt folivores are hindgut fermenters. Therefore, parts of the proximal colon and the caecum are enlarged and provide the major sites for fermentation. The caecum is adjacent to the proximal colon, but separate from the normal flow of digesta. Digesta are roughly separated by size in the proximal colon and small particles and solutes are retained in the caecum while large particles are excreted relatively quickly (Hume 1982). This is the case in koalas, ringtail possums and greater gliders. In the brushtail possum, however, the caecum functions as an extension of the proximal colon, resulting in a single fermentation chamber. Thus, selective separation of digesta does not occur in the brushtail possum (Hume 1982).

The caecum and proximal colon wall of the koala are colonised with a tannin-protein-complex-degrading enterobacteria, which hydrolyses protein-hydrolysed tannin-complexes, releasing nitrogen as ammonia, which is then available for microbial protein synthesis in the hindgut which, in turn, is likely to be a valuable source of nitrogen for the koala (Osawa *et al.* 1993). Juvenile koalas ingest pap, a special faecal deposition of caecum contents, at the time of weaning (Lee and Martin 1988). It has been hypothesised that this inoculates the gastrointestinal tract with symbiont bacteria (Osawa *et al.* 1993). There is no report of whether this type of bacteria colonises the colon and caecum of the other eucalypt folivores.

Cork and Foley (1991) hypothesised that the evolution of the fermentation processes, utilising cell wall components, in the caecum-colon is the most appropriate adaptation for small mammals utilising mature tree foliage as their main food source. This digestive structure also allows absorption of PSMs in the upper gut. This prevents the microbial organisms responsible for fermentation being

exposed, thus protecting them from the antimicrobial properties of PSMs such as terpenes (Nagy *et al.* 1964; Freeland and Janzen 1974; Foley *et al.* 1987).

1.4.2.2. Dentition

The teeth of folivorous mammals have evolved such that mastication of foliage prepares the food for further digestion. The highly lignified fibre in eucalypt leaf requires the teeth to perform a cutting action rather than crushing (Sanson 1980). Therefore the teeth have evolved to efficiently cut and grind leaf into a paste like digesta containing small particles ready for further digestion, absorption of cell contents and fermentation of cell wall components (Martin and Handasyde 1995).

1.5. Pharmacokinetics of plant toxins in marsupials

As mentioned before, little is understood about the metabolism and detoxification of PSMs, and monoterpenes in particular, in marsupial eucalypt folivores. Consideration of pharmacokinetic parameters involved in the metabolism of terpenes and other simple PSMs follows.

1.5.1. Absorption

Passive absorption of monoterpenes through the epithelium of the stomach and small intestine is likely considering the size and lipophilicity of monoterpenes. Terpenes have relatively low molecular weights (130 - 154) and are highly lipid soluble. Experimental evidence of rapid and extensive absorption has been reported. Southwell *et al.* (1980) found that when brushtail possums were dosed with α- and β-pinene, 1,8-cineole and *p*-cymene only trace amounts of distillable oil was present in the faeces. Similarly Foley *et al.* (1987) reported 96 - 97 % of oils were absorbed from the stomach and proximal small intestine of the greater glider and the brushtail possum. Likewise, less than 15 % of ingested oil was excreted in a volatile form from faeces of koala fed a diet of *E. punctata* (Eberhard *et al.* 1975). These values may be high since the oils present in the faeces were not always the same as the leaf oils. Alteration of the oil composition would suggest biliary secretion of hepatic metabolites or microbial metabolism.

1.5.2. Distribution

The lipophilic nature of absorbed terpenes suggests that they would be rapidly distributed into fatty tissue around the body. There is pharmacokinetic evidence of this occurring after terpene absorption in humans. Biphasic elimination for plasma could be consistent with a rapid distribution into fatty tissues followed by slower elimination by metabolism (Stimpfl *et al.* 1995; Jager *et al.* 1996).

1.5.3. Metabolism

After absorption, lipophilic compounds enter the general blood stream and are available to cause toxicity to organs and tissues. Metabolism of lipophilic compounds, such as terpenes, to more polar and water soluble metabolites facilitates their elimination via renal or biliary excretion (Alvares and Pratt 1990). From the extensive reports on terpene metabolism in eutherian mammals and the limited work on metabolism in marsupials, it is evident that two phase metabolism is utilised in marsupials. Phase I reactions alter the functional groups of the compound by oxidation, reduction or hydrolysis. These reactions increase polarity by either adding polar functional groups, eg. by hydroxylation, or removing non-polar groups, eg. by demethylation (Alvares and Pratt 1990). Phase II reactions further increase polarity by forming a conjugate of the xenobiotic with an endogenous polar molecule such as glucuronic acid, glutathione or glycine (Caldwell 1982). Both types of reaction occur predominantly in the liver and are catalysed by complex enzyme systems.

1.5.3.1. Phase I metabolism reactions

Data are now being accumulated on xenobiotic metabolising enzyme systems in marsupials. Receiving most attention is the cytochrome P450 (CYP) enzyme family, located on the endoplasmic reticulum, and therefore isolated in the microsomal fraction of mammalian liver. They catalyse aliphatic oxidations and aromatic hydroxylations of terpenes in mammals, resulting in alcohol and hydroxy metabolites (Alvares and Pratt 1990; G. Pass personal communications). Early work reported that wild marsupials (including three ruminant herbivores, an omnivore and the hind-gut fermenter, the common brushtail possum) have a low liver to body weight ratio, but liver CYP content was comparable with eutherians (McManus and Ilett 1977). Investigations of other tissues (kidney, lung and gastrointestinal) revealed minimal extra-hepatic CYP activity. These authors also reported that the metabolising capacity of marsupials, as determined by their ability to metabolise three specific substrates (aniline, benzopyrene and ethylmorphine), was less than in the rat (McManus and Ilett 1977).

Recent *in vitro* work has examined in more detail the CYP enzyme system of brushtail possums (Bolton and Ahokas 1997; Olkowski and Gooneratne 1998; Pass *et al.* In press). CYP content and activity have been shown to be associated with diet (Bolton and Ahokas 1997; Pass *et al.* In press). In particular, possums maintained on a controlled diet containing eucalypt terpenes (1,8-cineole, *p*-cymene, α-pinene and limonene) were shown to have induced CYP enzymes resulting in increased metabolism of a number of standard test substrates when compared to possums maintained on a terpene free diet (Pass *et al.* In press). Induced metabolism of test substrates indicated that brushtail possums possess terpene inducible hepatic CYPs that correspond to several CYP families in the rat (CYP2C6, 2C11 and 2E1). Olkowski and Gooneratne (1998) demonstrated *in vitro* inhibition of specific substrate reactions with imidazole compounds, with clotrimazole and miconazole causing the most potent inhibition.

Preliminary *in vitro* investigations into koala hepatic microsomal activity found that although the CYP content was comparable to rats, overall enzyme activity was lower

in the koala (Stupans *et al.* 1999). Test substrates indicate that koalas possess hepatic CYPs that correspond to several CYP families (CYP2A, 2B, 2C, 2E and 3A). However, without data to relate rat and koala CYPs (and to marsupials in general), it cannot be said which CYPs are active in the koala (Stupans *et al.* 1999).

Rat liver CYP isozymes have also been induced after exposure to α-pinene and 1,8 cineole (Jori *et al.* 1969; Jori *et al.* 1972; Jarvisalo and Vainio 1980). More recently, the CYP isozymes induced by 1,8-cineole in the rat have been identified *in vitro* as CYP2B1 and 3A2 and for α-pinene they have been identified as CYP2B1 and 4A2 (Hiroi *et al.* 1995). 1,8-Cineole has been shown to induce the metabolism of a number of substrates *in vitro* and the *in vivo* metabolism of pentobarbitone in rats (Jori *et al.* 1969). The metabolism of standard substrates (eg. aniline, aminopyrine and ethylmorphine) have also been shown to be induced by terpenes in brushtail possums (Bolton and Ahokas 1997; Olkowski and Gooneratne 1998; Pass *et al.* In press). However, the relevance of these standard substrates to the natural diet of possums is questionable. On the other hand, metabolic challenges from PSMs arise from the natural diet. Preliminary results suggest that the induction of CYPs caused by dietary terpenes in brushtail possums results in an increase in their own metabolism and elimination (G. Pass, University of Tasmania, personal communications).

Alcohol and aldehyde dehydrogenases are nonspecific enzymes which are unrelated to the CYP microsomal enzymes (Alvares and Pratt 1990), yet, may be equally important in the oxidation of terpene metabolites, transforming alcohol products of the CYPs, into carboxylic acid metabolites. The dehydrogenase enzymes are found in the cytosolic fraction of cells and oxidise a number of endogenous and exogenous alcohols, such as vitamin A and ethanol, first to their aldehyde and then to their carboxylic acids, respectively. Although this enzyme system has yet to be investigated in marsupials, these enzymes are likely to be important in the metabolism of terpenes.

Microsomal CYP metabolism of ethanol to acetic acid has been demonstrated in humans and experimental animals after chronic ingestion (Lieber 1999). Whether a similar microsomal alcohol oxidising system is induced in marsupials after chronic ingestion of terpenes requires investigation.

1.5.3.2. Phase II metabolism reactions

Conjugation of exogenous (and endogenous) compounds, or their metabolites, with polar endogenous molecules such as glucuronic acid, glycine and glutathione serve to further increase renal or biliary excretion (Caldwell 1982).

Glucuronidation is utilised by many animals due to its high capacity and its effectiveness in increasing the renal excretion of xenobiotics and endogenous waste compounds (Hirom *et al.* 1977; Caldwell 1982). Glucuronyl transferases are responsible for catalysing these reactions and, like the microsomal oxidases, are a complex family of enzymes. Brushtail possums have a low capacity to form sulfate conjugates (probably due to dietary deficiencies of sulfate; Roy 1963), and therefore, the glucuronidation pathway becomes more important (Baudinette *et al.* 1980).

Hinks and Bolliger (1956 and 1957) noticed that folivorous marsupials presented high levels of glucuronic acid or glucuronides in their urine compared to other marsupials. Yet, McLean *et al.* (1993) found glucuronidation of terpenes, derived from dietary *E. radiata*, to be minimal in ringtail possums, despite elevated urinary glucuronic acid concentrations. When fed *E. radiata* for extended periods, the excretion of glucuronides increased over the first three days, but remained less (30 %) than the total amount of oxidised terpene metabolites. This suggests that for at least this species of eucalypt folivore, glucuronic acid is not necessarily involved in the elimination of terpenes.

The glycine conjugation pathway is active in many marsupial species, as the glycine conjugate of benzoic acid, hippuric acid, is commonly excreted (Awaluddin and McLean 1985). A glycine conjugated metabolite of *p*-cymene has been reported in the koala (Southwell 1974).

Finally, conjugation of strongly electrophilic substrates with the tripeptide glutathione allows the detoxification of a wide range of environmental xenobiotics. Recently a single glutathione transferase enzyme was isolated from the hepatic cytosol of the brushtail possum (Bolton and Ahokas 1997). Substrates for this enzyme have yet to be identified, but are likely to be from lipid and nucleic hydroperoxides, formed during oxidative xenobiotic metabolism (Bolton and Ahokas 1997).

1.5.4. Excretion

Metabolites of plant toxins may be eliminated from the body via the kidneys or by biliary excretion. The kidneys play a major role in the elimination of both endogenous and exogenous metabolites via several processes (Rowland and Tozer 1989). Firstly, metabolites can be passively filtered from the blood into the urine. Secondly, organic anions can be actively secreted into the urine via the organic acid transport system. Secretion of organic acids via the organic acid transport system allows clearance rates equal to renal plasma flow. It has been demonstrated that the physiological mechanism of the organic acid transport system of the brushtail possum is similar to that of eutherian mammals (Miller and Morris 1982). Finally, the elimination of metabolites may be further influenced by tubular reabsorption from the urine back into the blood.

Biliary excretion is important in the elimination of metabolites and their conjugates with molecular weights greater than 400 - 450. Glucuronide conjugates of both endogenous and exogenous compounds are often above the molecular weight threshold and may be excreted via this route (Caldwell 1982). Although the molecular weights of oxidised terpene metabolite glucuronides are borderline for biliary secretion, the glucuronide of at least one terpene, *l*-menthol, is excreted by this route (Madyastha and Srivatsan 1988; Yamaguchi *et al.* 1994).

1.6. Eucalyptus oil toxicity

Toxicity of terpenes and *Eucalyptus* oil in particular have been reported, in humans, at doses per body weight which are only a fraction of those ingested daily by marsupial eucalypt folivores. Doses of *Eucalyptus* oil causing morbidity or death are highly variable. Death has resulted from a dose of *Eucalyptus* oil as small as 3.5 ml and yet recovery from a dose as large as 120 - 220 ml has been reported (Gurr and Scroggie 1965).

Toxic signs for rats dosed with *p*-cymene and 1,8-cineole during determinations of LD_{50} included depression soon after dosing, bloody lacrimations, diarrhoea, irritability and coma plus prolonged scrawny appearance in surviving rats (Jenner *et al.* 1964). Similarly, signs of CNS depression are evident after ingestion of *Eucalyptus* oil in humans. Other symptoms of poisoning include epigastric burning, nausea and vomiting, dizziness and muscular weakness, diminution or loss of reflexes, miosis, tachycardia and a feeling of suffocation, cyanosis, nephrotoxicity, delirium, convulsions and depression of consciousness which may progress to coma and death (Patel and Wiggins 1980; Martindale 1993; Webb and Pitt 1993; Whitman and Ghazizadeh 1993; Barnes 1996).

Toxicity is presumably due to the unmetabolised oils, which are lipophilic and therefore readily cross the blood brain barrier. Although the mechanisms of toxicity are unknown, active metabolites may be involved as in the case of pulegone and limonene (Thomassen *et al.* 1991; Nelson *et al.* 1992).

1.7. Applications of plant-herbivore interactions

Understanding the complex interactions between plants and herbivores provides fundamental information on the biology of the respective species. In particular understanding dietary selection is important in the long term conservation of vulnerable species and their habitats. Furthermore, where there is conflict between native fauna and human activities, such as herbivory on forestry and farming industries, knowledge of metabolism capacities and associated diet selection may allow the development of specific cultivars which would deter native fauna, a more acceptable method of control than direct poison, laid as baits.

PART 1

COMPARATIVE METABOLISM OF *P*-CYMENE IN FOUR MARSUPIAL FOLIVORES AND THE RAT

CHAPTER 2

METHOD DEVELOPMENT OF *P*-CYMENE METABOLITE ASSAY

2.1. Introduction

Part 1 of this thesis compares the metabolic fate of *p*-cymene, a common component of many *Eucalyptus* oils, in four marsupial eucalypt folivores and the laboratory rat. The marsupial species studied cover a range of browsing herbivore niches as discussed in Chapter 1 - section 1.3.

The metabolic fate of *p*-cymene was studied by quantifying urinary metabolites excreted by each species. The development of the assay for quantifying urinary metabolites is detailed in this chapter. Metabolites were identified predominantly by comparison with published mass spectra. Standards for major metabolites, excreted by each species, were isolated from the respective urines in sufficient quantities for preparation of calibration curves allowing quantitation. Development of the assay was mostly carried out using urine from rats, due to its ready availability. However, validation of some methods and reproducibility studies were performed using urine from other species. In each case, the species used is noted. Experimental details specific for individual species are detailed in the appropriate chapters.

Chemical structures and names of metabolites are reported in Figure 2.1 and Table 2.1.

Animal husbandry and dosing methods for each species are described in the respective chapters (Chapters 3 to 7). Apart from the koala, each species was given *p*-cymene by oral gavage. The rats, brushtail possums and koalas received two doses of *p*-cymene each (0.37 and 1.49 mmol/kg) while greater gliders and ringtail possums received only one dose each of 1.49 mmol/kg and 0.37 mmol/kg, respectively. These doses were chosen to examine the dose-dependence of *p*-cymene metabolism. They represent a conservative estimate of terpene intake by brushtail and ringtail possums eating a *Eucalyptus* diet. Furthermore, they are, respectively, half and double the 100 mg/kg dose used safely in rats and guinea pigs by previous investigators (Walde *et al.* 1983). For each animal, pre- and post-dose collections of urine and faeces were made.

Approval for experimental work involving rats, brushtail and ringtail possums was granted by the University of Tasmania Ethics Committee (Animal Experimentation; Approval number 95053). Parks and Wildlife Service, Department of Environment and Land Management, Tasmanian State Government granted approval for studies involving brushtail and ringtail possums (Approval number FA 96223). Experiments using greater gliders and koalas were performed in collaboration with Dr W. J. Foley, Australian National University, ACT (formerly of James Cook University, Townsville, Queensland). For each species ethics approval was granted by the appropriate institutions. All animal experimental procedures complied with the Australian Code of Practice for Care and Use of Animals for Scientific Purposes.

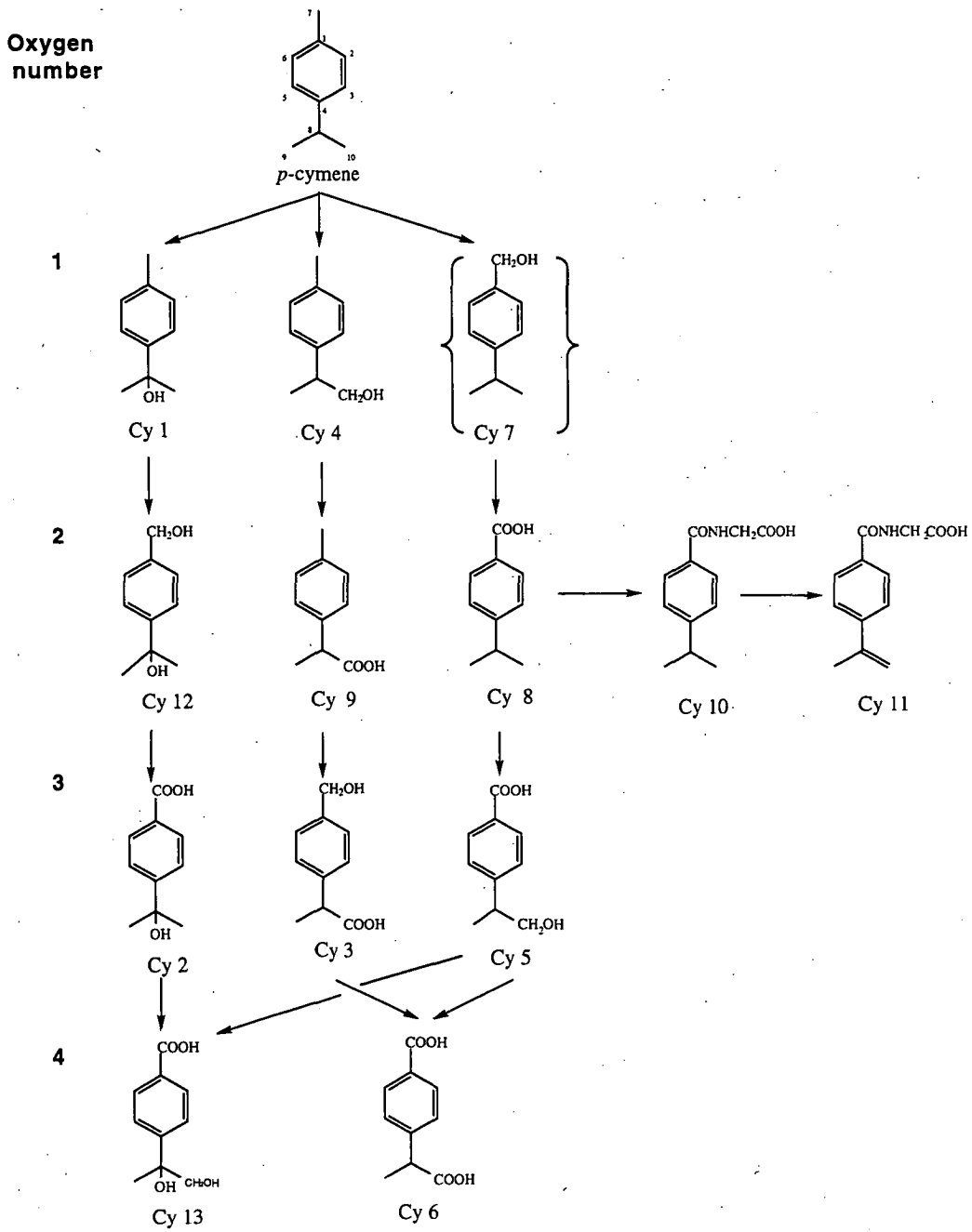


Figure 2.1. Chemical structures of *p* -cymene metabolites and possible metabolic pathways in all five species. All metabolites identified are shown in their underivatised forms. Metabolites are grouped according to the number of oxygen atoms acquired during oxidation (marked on the left hand side of the figure). Cy 7 was not found in this study but was a key precursor metabolite and is shown for the sake of completeness. Chemical names for each metabolite are recorded in Table 2.1.

Table 2.1. Assigned and chemical names of *p* -cymene metabolites found in all species studied. Literature references of published mass spectra are given. The derivatisation of metabolites is also reported and whether the metabolite was quantified.

Assigned name	Chemical name	Derivative[1]	Quantified Y/N	Purity[2] %	References
Cy 1	2-*p*- tolypropan-2-ol	M	Y	92.7	Ishida *et al* ., 1981; Walde *et al.*, 1983
Cy 2	2-*p*- carboxyphenylpropan-2-ol	M	Y	91.6	Ishida *et al* ., 1981; Walde *et al.*, 1983
Cy 3	2-*p*- hydroxymethylphenylpropionic acid	M	N	85.0	Walde *et al.*, 1983
Cy 4	2-*p*- tolypropan-1-ol	M	N	-	Ishida *et al.*, 1981
Cy 5	2-*p*- carboxyphenylpropan-1-ol	M	Y	96.0	Walde *et al.*, 1983
Cy 6	2-*p*- carboxyphenylpropionic acid	M	Y	95.0	Walde *et al.*, 1983
Cy 8	*p*- isopropylbenzoic acid (cumic acid)	M	Y	99.0	Ishida *et al.*, 1981; Walde *et al.*, 1983
Cy 9	2-*p*- tolypropionic acid	M	Y	-	Ishida *et al.*, 1981; Walde *et al.*, 1983
Cy 10	*p*- isopropylbenzoylglycine (cuminuric acid)	M	N	-	Walde *et al.*, 1983
Cy 11	*p*- isopropenylbenzoylglycine	M	N	-	Walde *et al.*, 1983
Cy 12	2-*p*- (hydroxymethyl)phenylpropan-2-ol	TMS	N	-	Walde *et al.*, 1983
Cy 13	4-(1,2-dihydroxy-1-methylethyl)-benzoic acid (2-*p*- carboxyphenylpropan-1,2-diol)	M+TMS	Y	-	

[1]M = methylated, TMS = trimethylsilyl ether.

[2]Purity of typical standards used for quantitation were estimated by GC analysis. In some cases metabolites were isolated on more than one occasion. The measured purity of isolated metabolites for each species are reported in the relevant chapters.

Cy 8 was purchased commercially and Cy 9 was quantified using the Cy 8 calibration curve. Cy 13 was quantified using the Cy 5 (M+TMS) calibration curve.

2.2. Methods

2.2.1. Extraction and derivatisation of urine samples

The assay of urinary terpene metabolites was derived from that of McLean *et al.* (1993). Urine was analysed for both free (unhydrolysed) and total (hydrolysed) metabolites.

Urine samples were thawed and 1 ml samples placed into tapered centrifuge tubes. In some cases the metabolite concentration was too strong and required dilution (0.5 ml urine with 0.5 ml distilled water). The urine was acidified to pH 1 using 40 µl 5 M hydrochloric acid (HCl). Preliminary trials comparing different extraction procedures found that after three sequential washes (1 x 2 ml and 2 x 1 ml) of ethyl acetate extracted > 99 % of metabolites from urine (Table 2.2). Vortexing for 1 min and subsequent centrifuging at 2000 rpm for 5 min ensured thorough mixing and separation of ethyl acetate and urine.

Table 2.2. Efficiency of extracting metabolites from urine with sequential washes with ethyl acetate (1 x 2 ml and 3 x 1 ml; n = 3).

Extraction	Percent of total metabolite extracted			
#	Cy 1	Cy 2	Cy 5	Cy 6
1	65.5	81.4	86.2	82.1
2	27.7	17.2	13.8	16.7
3	6.1	1.3	0.0	1.2
4	0.7	0.0	0.0	0.0

The ethyl acetate extracts were stored in 5 ml glass screw capped vials. To enhance the chromatography of polar carboxylic acid metabolites, the urine extracts were derivatised with diazomethane, to form their respective methyl esters. One ml ethyl acetate extract was transferred to a clean vial placed in an ice bath inside a fume hood. Approximately 1 ml of ethereal diazomethane was added to each vial. Vials were capped and the reaction mixture allowed to sit for 30 min. Excess diazomethane was evaporated and the sample concentrated to about 0.5 ml under a stream of nitrogen in a heating block held at 25 - 30°C. The samples were then ready for analysis by gas chromatography (GC).

Preparation of diazomethane. Diazomethane was prepared in the laboratory and was an adaptation of the methods described by Schwartz and Bright (1974) and Aldrich (1983). All glassware had smooth glass fittings to minimise risk of explosion of diazomethane. One gram of N-methyl-N-nitroso-*p*-toluene sulphonamide (Diazald; Aldrich Chemical Co., Milwaukee, Wisconsin, USA) was dissolved in 20 ml diethyl ether in a round bottomed flask. Five ml of ethanol (98%) was measured and 0.6 ml 40 % aqueous potassium hydroxide added. The ethanol mixture was added to the ether mixture and the contents heated in a water bath at 60 - 70°C. Ethereal diazomethane was distilled into a collection flask and chilled in ice. The resulting ethereal mixture contained approximately 0.2 mmol/ml diazomethane. Diazomethane

was stored at -18°C in a sealed flask inside a screw cap jar, containing benzoic acid. The benzoic acid was included to react with any escaping diazomethane to form methyl benzoate.

Total metabolite content of urine was measured by hydrolysing the urine sample prior to extraction. Both acid and enzyme hydrolysis methods were trialed as described in Section 2.2.5.3.1. Enzyme hydrolysis using extract of *Helix pomatia* (ß-glucuronidase plus aryl sulphatase; Boehringer Mannheim, Germany) proved to be the most effective method. Urine was buffered to pH 5.3 by adding 0.2 ml 1.1 M acetate buffer. Then 100 µl enzyme extract was added and the mixture incubated overnight at 37.5°C.

After incubation the samples were treated in the same manner as free metabolite analyses. Emulsions tended to form in the ethyl acetate phase and required physical disruption by stirring with a clean pipette tip and re-centrifuging. The effect of the enzyme extract on the recovery of metabolites is considered in Section 2.2.5.3.2.

Hydrolysed pre- and post-dose urine extracts were also derivatised with N,O-bis (trimethysilyl) trifluoroacetamide (BSTFA; Alltech Associates Inc, Deerfield, Illinois, USA) to produce trimethyl silyl (TMS) derivatives of hydroxy and carboxy functional groups. Only one metabolite (Cy 12) was identified as its TMS derivative alone and it was not quantified as it was a trace metabolite. TMS derivatisation allowed comparisons with published studies of TMS derivatised metabolites (Walde et al. 1983). To derivatise ethyl acetate extracts, 20 µl of sample was placed into a 100 µl GC autosampler vial insert and evaporated to dryness under a stream of nitrogen at 50°C. Twenty µl BSTFA was then added and the tubes capped and heated at 60°C for 20 min prior to analysis by GC.

A very polar metabolite (Cy 13) was identified for the first time in koala urine after the urine extract was doubly derivatised with both diazomethane and BSTFA.

2.2.2. Gas chromatography

Three instruments were used for gas chromatography throughout the analyses. The majority of the method development and some quantitative analyses used a Varian 3300 Gas Chromatograph fitted with a Varian 1077 Split/Splitless Capillary Injector, Flame Ionisation Detector (FID) and Star Workstation (Version A.2, Varian Pty Ltd, Walnut Creek, California, USA).

Quantitative analyses for the rat and brushtail possum urine used a Hewlett Packard 5880A series GC fitted with an FID detector and a Hewlett Packard 7671A automatic sampler. Chromatographs were recorded on a Hewlett Packard 5880A series GC terminal integrator (Hewlett-Packard Australia Ltd., Melbourne, Australia).

On the above instruments, chromatography was carried out on a 25 m HP-5 capillary column (0.52 mm ID, coated with 0.25 µm crosslinked 5% phenyl methyl silicone). The column was shortened to 20 m to allow the remaining 5 m to be cut into 50 cm lengths which were used as pre-columns, joined to the main column by a mini-union. Pre-columns were replaced periodically to enhance chromatography and

to extend the life of the capillary column. GC conditions were: injector 250°C, split ratio 1:40 for the Varian GC and 1:20 for the Hewlett Packard GC; carrier He (9 psi), oven 120 - 190°C at 5°C/min, 190 - 290°C at 30°C/min, 290°C for 2 min, detector 300°C.

Metabolites were identified using a Hewlett-Packard (HP) 5890 gas chromatograph and HP 5970B mass-selective detector (GC/MS) with HP 59970A Chemstation software (Hewlett-Packard Australia Ltd., Melbourne, Australia). The Chemstation software was modified to enable exporting of mass spectra to the NIST MS Search Program (NIST 1998), for searching both the NIST and custom made terpene metabolite libraries of mass spectra (see section 2.2.3). GC/MS chromatography was carried out on a 25 m HP-1 capillary column (0.32 mm ID, coated with 0.52 µm crosslinked 1% phenyl methyl silicone). GC/MS operating conditions were: splitless injector, 250°C, detector, 300°C, oven, 60 - 190°C at 5°C/min, then 190 - 290°C at 30°C/min and held at 290°C for 5 min, carrier gas He at a pressure of 12 psi. Mass spectrometry (MS) conditions required a solvent delay of 2 min, and a mass range of 40 - 450 was selected by using 1.5 scans/sec.

The same instrument was used for analysing koala urine extracts. Since *p*-cymene metabolites were overwhelmed by other leaf components, it was necessary to extract mass chromatograms of diagnostic ions to separate some metabolites. Koala urine extracts were analysed as both methylated derivatives and methylated plus TMS derivatives (see Chapter 7 - section 7.2.3). When analysing the doubly derivatised extracts, the MS required an extended solvent delay of 6 min.

The chromatography conditions resulted in good separation of *p*-cymene derived peaks for all species. Figure 2.2 is an example of the chromatography for both pre- and post-dose hydrolysed rat urine samples. Chromatography of the relatively polar metabolite, Cy 5, was sensitive to deterioration of column function. For the rat, brushtail possum, ringtail possum and greater glider analyses, this was rectified by replacing the pre-column of the capillary column as required. For koala urine analyses, Cy 5 was analysed as the methyl ester plus TMS derivative, which improved its chromatography.

2.2.3. Identification of metabolites

Identification of metabolites was assisted by a customised library of mass spectral data. All encountered published mass spectra, pertaining to terpenes and their metabolites, were transcribed into a format acceptable to the NIST Library programme. Also included in the library were the mass spectra of all terpene metabolites identified throughout the course of the project. The aim was to develop a comprehensive library capable of identifying as many terpene metabolites as possible. Specific *p*-cymene metabolite mass spectral data have been published by a number of other researchers (Southwell *et al.* 1980; Ishida *et al.* 1981; Walde *et al.* 1983; Ishida *et al.* 1989).

Mass spectra derived from *p*-cymene metabolites were compared, by using probability based comparisons, to the mass spectral data in the customised library using the NIST Library programme. Searching unknown metabolites against the mass spectra in this library resulted in the identification of all metabolites, except Cy 13.

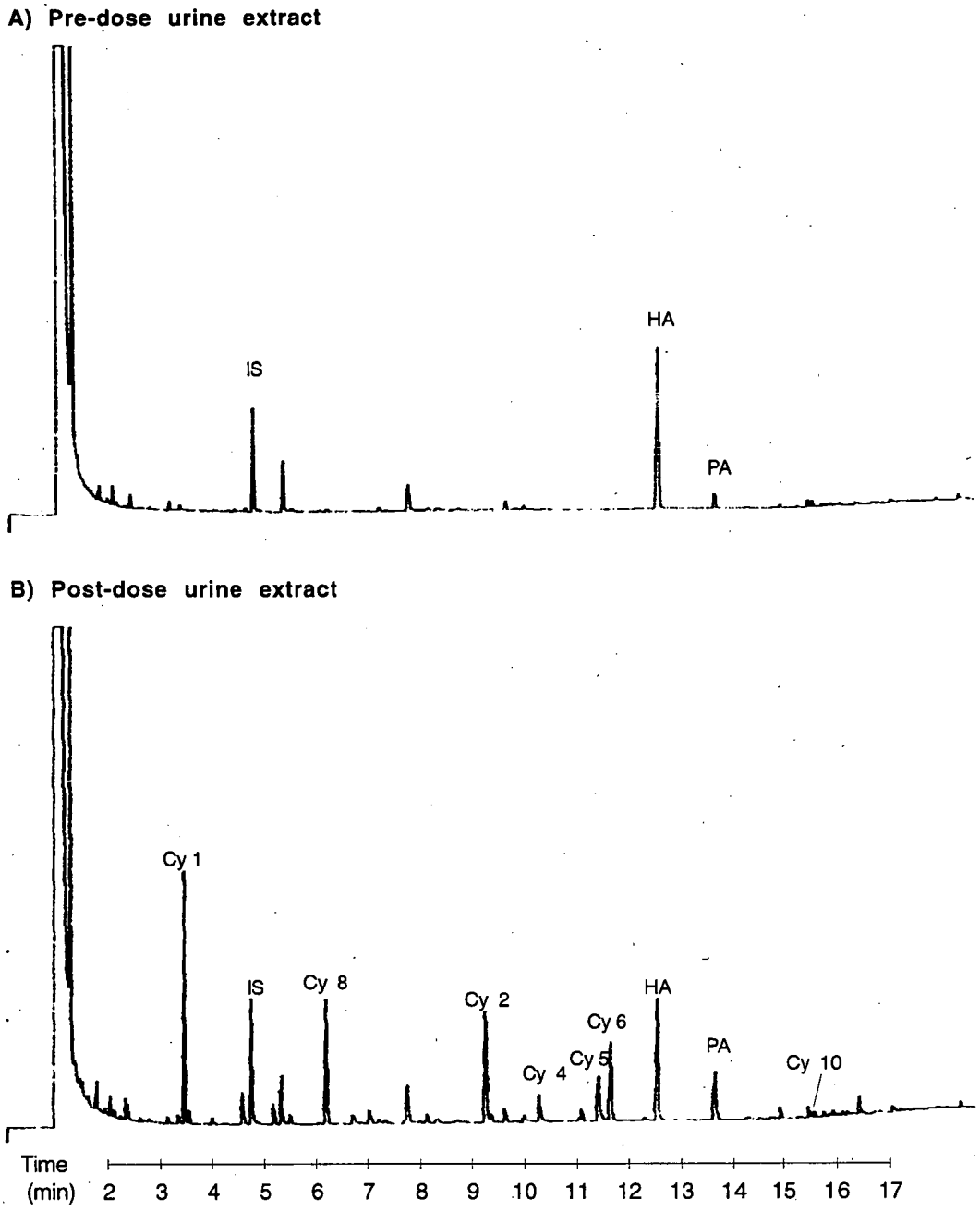


Figure 2.2. Chromatograms from GC/FID of hydrolysed rat urine extracts comparing A) pre-dose and B) post-dose samples. Urine extracts were methylated for chromatography. IS = 2,5-dimethyl benzoic acid, HA = hippuric acid, PA = phenyl acetic acid. Chemical structures and names of *p* -cymene metabolites are reported in Figure 2.1 and Table 2.1.

Searching total ion current (TIC) chromatograms of urine extracts, for diagnostic ions of all metabolites reported by Walde et al. (1983), failed to identify some of the minor metabolites reported.

2.2.4. Isolation of metabolites

Quantitation of metabolites required calibration standards. Only one metabolite, cumic acid (Cy 8; Figure 2.1), was available commercially (4-isopropyl benzoic acid; Aldrich Chemical Co., Milwaukee, Wisconsin, USA). Cumic acid was also used to quantify the structural isomer Cy 9 (Figure 2.1). Cy 10 was synthesised to allow its quantitation in koala urine (Chapter 7 - section 7.2.4). All other major metabolites were isolated from the urine, usually from each species in which the metabolites were present. However, the greater glider and koala were fed on *Eucalyptus* leaves throughout the dosing experiments and *p*-cymene metabolites were overwhelmed by leaf derived metabolites. Fortunately, the metabolites excreted by these two species were available from previous work or could be re-extracted from excess brushtail possum urine. The exception to this was the major metabolite found in koala urine (Cy 13) which is considered in detail in Chapter 7 - section 7.2.5.

The first step in the isolation process was to extract the metabolites from a large volume of urine. Approximately 65 ml of combined rat urine, which had been collected in the first 24 h after the *p*-cymene dose of 1.49 mmol/kg, was placed in a separating funnel. The urine was acidified to pH 1 using 3 ml 5 M HCl and extracted with three sequential washes of ethyl acetate (1 x 100 ml and 2 x 50 ml). The ethyl acetate extract was concentrated to approximately 10 ml using a rotary evaporator in a water bath at 40°C.

The concentrated extract was methylated by adding diazomethane until a permanent yellow colour was maintained (approximately 30 ml). The flask was stoppered and allowed to sit for 30 min in an ice bath. Excess diazomethane was evaporated using a flow of nitrogen.

Preparative thin layer chromatography (pTLC) provided a preliminary clean up and separation of metabolites. pTLC conditions were modified to produce good separation of metabolites, while leaving many unwanted urinary compounds at the origin.

Initially, small analytical TLC plates were used to optimise conditions and to identify bands of separation occurring on the plates. The plates used were pre-coated aluminium sheets of silica gel with a fluorescent marker (60-F_{254}, gel thickness 0.2 mm, 20 x 20 cm, DC-Alufolien, Kieselgel, Merck, Germany) and the mobile phase was ethyl acetate/hexane, 50:50. *p*-Cymene metabolites quenched fluorescence under ultraviolet (UV) light, facilitating their detection on the plates.

Metabolite zones were scraped from the plates and redissolved with two sequential washes of methanol (3 and 1 ml respectively). The methanol and silica gel were separated by centrifugation at 2000 rpm for 5 min. The methanol extract was decanted into a round bottom flask and methanol evaporated using a rotary evaporator (40°C). The solutes were redissolved in 1 ml ethyl acetate and re-centrifuged to separate any remaining silica before GC analysis. Metabolites and endogenous compounds in each zone were identified by their GC/FID retention times and mass spectra (GC/MS) when required.

To isolate sufficient quantities of metabolites for quantitation purposes, the conditions were transferred to pTLC. Glass plates (approximately 75 cm wide) were coated with powdered silica gel (60 GF 254, Keiselgel, Merck, Germany). A slurry of 70 g of silica and 140 ml of water was prepared and applied along the length of the plate using a TLC gel applicator. The plates were activated overnight at 110°C. Derivatised urine extract was applied to the plates using a custom built mechanical applicator (Chemistry Department, University of Tasmania). The plates were developed in large glass tanks saturated with mobile phase. Plates were allowed to dry at room temperature after developing.

Ten zones were removed according to the bands identified from the analytical plates and by quenching of fluorescence under UV light (Figure 2.3). Each zone was redissolved in sequential methanol washes (1 x 30 ml and 2 x 15 ml) which were then evaporated to dryness. The residues were redissolved in ethyl acetate and analysed by GC to confirm the location of each metabolite.

Either a second pTLC development or semi-preparative high performance liquid chromatography (pHPLC) was used to further purify the metabolites. Rat and brushtail possum metabolite isolates were further purified by a second pTLC application using the same conditions described above. Greater glider and ringtail possum metabolite isolates were further purified using pHPLC. pHPLC was the preferred method for the secondary purification of the metabolites, however a suitable HPLC pump was not always available.

The HPLC conditions were optimised using a Varian Star 9010 Solvent Delivery System coupled to a Varian Star 9050 Variable Wavelength UV-VIS Detector fitted with a Waters NovaPak C18 HPLC column (3.9 x 150 mm) and a 10 µl Rheodyne injection port. The mobile phase used was water/methanol 45:55 and a flow rate of 1 ml/min. The detector used a wavelength of 210 nm. HPLC peaks were identified by collecting the output of each peak. The solvent was evaporated and extra methanol added to azeotrope the remaining water from the mobile phase. The sample was then redissolved in ethyl acetate and analysed by GC/FID to confirm the identity of metabolites.

The analytical conditions were transferred to the pHPLC. A Shimadzu HPLC instrument was used, with a DGU-4A degasser, two LC 9A pumps, and a SPD-10A UV-VIS detector (Shimadzu Scientific Instruments Inc., Australia) fitted with a 1 ml injection loop (Rheodyne Incorporated, Cotati, California, USA). The column was a 25 x 100 mm cartridge (prep Nova-Pak HR C18 6 µm 60 Å) with a Guard-Pak Cartridge guard column (Waters Pty Ltd, Milford, Massachusetts, USA). The column was radially compressed in a Waters PrepLC 25 mm Module at < 1500 psi. The mobile phase was methanol/water (45:55), flow rate 8 ml/min. Peaks were detected by UV absorbance (210 nm), collected, the solvent removed by rotary evaporation, and the residue dried under vacuum.

The identities of the isolated metabolites were confirmed by GC retention times and GC/MS. Purity of isolated metabolites determined by GC was > 90%, except for Cy 3 which was 85% (Table 2.1). None of the impurities interfered with quantitative analyses. The weight of each metabolite was corrected for its percentage purity in the preparation of calibration curves.

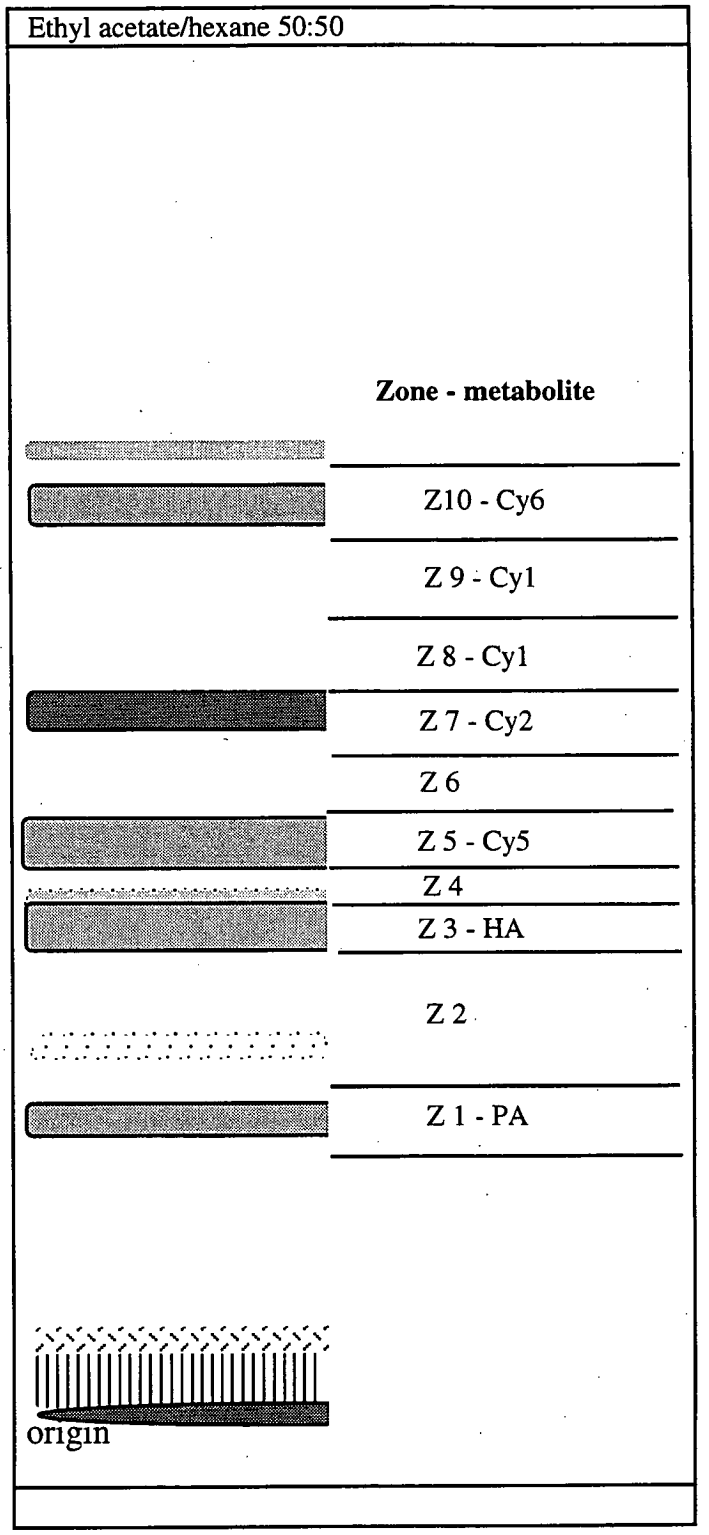


Figure 2.3. Diagramatic representation of developed pTLC plate depicting the separation of methylated *p* -cymene metabolites from rat urine during the process of isolating urinary metabolites for quantitation. Bands were identified by quenching of fluorescence under UV light and zones removed are indicated on the right hand side, along with the metabolites occurring in respective zones. pTLC conditions are described in section 2.2.4.

2.2.5. Quantitation of *p*-cymene metabolites

2.2.5.1. Calibration curves for *p*-cymene metabolites

The metabolites isolated for quantitation depended on the major metabolites excreted by each species. Metabolites Cy 1, 2, 5, 6, 8, 9, 10 and 13 were all quantified in at least one species. Of these metabolites, Cy 2, 5, 6, 8, 9 and 13 were carboxylic acids, Cy 1 a secondary alcohol and Cy 10 the glycine conjugate of cumic acid (See Figure 2.1. and Table 2.1.). To improve chromatography and to facilitate isolation, carboxylic acid metabolites were derivatised to their methyl esters. However, it is required that standards used for quantitation be the same as those excreted in the urine (ie. the underivatised metabolites). Metabolites which had been isolated as their methyl esters therefore needed to be "underivatised" to return them to their respective carboxylic acids. The standards were then subject to the same workup as the urine samples from dosed animals. Saponification was used to hydrolyse methyl ester metabolites (Section 2.2.5.1.2). To avoid confusion, calibration curves were calculated as molar concentrations.

Cy 1 is a secondary alcohol and therefore does not methylate. It was added to the calibration curve dilutions after the saponification process as it was relatively volatile.

2.2.5.1.1. Stock solutions of metabolites

Metabolites were accurately weighed (1 - 5 mg) into an autosampler vial and sealed with a teflon/rubber septum and screw cap. The metabolites were diluted with 400 µl methanol using an HPLC syringe.

Five dilutions of the stock solution were prepared, covering appropriate concentration ranges. Dilutions were made by measuring 10, 20, 30, 40, 50 and 50 µl respectively (the second tube of 50 µl was a control for monitoring the progress of saponification) into six centrifuge tubes and making the volumes up to 50 µl with methanol. Details of calibration curves for each species are reported in their respective chapters.

2.2.5.1.2. Saponification conditions

To conserve isolated metabolites, saponification conditions were established using the methyl esters of two commercially available carboxylic acids. 2,5-Dimethyl benzoic acid and 3-(3,4-dimethoxyphenyl) propionic acid (Figure 2.4) were the chosen models as they both had structural properties similar to *p*-cymene metabolites. The carboxylic acids were first methylated and then subsequently saponified with sodium hydroxide (NaOH) to determine optimal conditions for hydrolysis. Aliquots were taken from the mixture at timed intervals and analysed by GC. The disappearance of derivatised peaks from the chromatogram indicated complete hydrolysis.

Figure 2.4. Chemical structures of test carboxylic acids used for developing the method for saponification of methyl esters. **A)** 2,5-dimethyl benzoic acid is a relatively simple aromatic carboxylic acid (also used as the internal standard for quantitative analyses of urinary metabolites), and **B)** 3-(3,4-dimethoxyphenyl) propionic acid is an aliphatic carboxylic acid with a phenyl substituent.

Methods. A stock solution containing both carboxylic acids and internal standard (*p*-cymene; 0.1 mg/ml of each compound) in methanol was prepared. Two 2 ml samples were methylated using 1 ml of diazomethane. Complete methylation was confirmed using unmethylated acids for reference on TLC (mobile phase of ethyl acetate/hexane 50:50). After excess diazomethane had been evaporated, the methylated stock solution was diluted with water (1:3) to make an aqueous solution and a portion of this was extracted into ethyl acetate and analysed. GC/FID retention times (Rt) for 2,5-dimethyl benzoic acid and 3-(3,4-dimethoxyphenyl) propionic acid were 5.206 and 13.207 min respectively.

Two tubes of test carboxylic acid mixtures were diluted to represent methanol and acid concentrations comparable to actual calibration dilutions (approximately 0.15 mg/ml of each acid). Each tube of methylated carboxylic acids was alkalinised with 2 ml of 0.02 M NaOH. The amount of NaOH added was calculated from the molar amounts of methylated acid metabolites. One tube was kept at room temperature in the laboratory while the second tube was heated at 40°C.

The saponification progress was monitored by taking 200 µl aliquots from each tube after 0, 19 and 24 h. The aliquots were acidified with 20 µl 0.1 M HCl to pH 1 before extracting with 400 µl ethyl acetate and analysing by GC/FID. The absence of peaks on the chromatogram corresponding to the retention times of the derivatised acids indicated that saponification was complete within 19 h at 40°C.

2.2.5.1.3. Saponification of calibration dilutions

To hydrolyse the derivatised metabolites in the calibration dilutions, 0.5 ml 0.025 M NaOH solution was added to each dilution. Tubes were then heated at 40°C. Aliquots were taken from the control tube at 0, 2 and 4 h, acidified, extracted and analysed by GC/FID as described above. Complete hydrolysis had occurred within four hours.

2.2.5.1.4. Completion of calibration standards workup

Finally, 500 µl blank urine and internal standard (125 µg 2,5-dimethylbenzoic acid in 50 µl methanol) were added to each dilution (10 - 50 µl). The solutions were acidified, extracted and derivatised as described in Section 2.2.1 and calibration curves prepared.

Calibration curves prepared for the rat included the metabolite Cy 1. As previously mentioned, Cy 1 was added after saponification. To minimise the amount of methanol used in the calibration curve samples, Cy 1 dilutions were made with the internal standard. Therefore a stock solution of Cy 1 was made with 400 µl internal standard (2.5 mg 2,5-dimethyl benzoic acid per ml) and then 10 µl, 20 µl, 30 µl, 40 µl and 50 µl aliquots were added to the appropriate calibration dilutions and made to volume (50 µl) with additional internal standard (Section 2.2.5.1.1).

Within-day and between-day repeatability studies were performed on calibration dilutions prepared for greater glider urine analysis (Cy 2, 5 and 6). Five replicates of a high and a low concentration were analysed by GC/FID. An indication of the repeatability of calibration curves for each of the other metabolites (Cy 1, 5 (as the methyl ester plus TMS derivative), 8 and 10) was obtained opportunistically from between-day coefficients of variation (CoV) of low and high concentrations of calibration curve dilutions used for quantifying real samples. The CoVs are reported in Table 2.3. and were within acceptable limits of variation. All calibration curves were linear over the required concentration ranges. Calibration curves were prepared with each batch of urine samples analysed.

2.2.5.2. Quantitation of free *p*-cymene metabolites

Free metabolites were quantified by accurately measuring 1 ml of urine (or diluted urine when necessary) and adding internal standard (125 µg 2,5-dimethylbenzoic acid in 50 µl methanol). Samples were then extracted and analysed as described in Section 2.2.1.

2.2.5.3. Quantitation of total *p*-cymene metabolites

Total metabolites were measured by hydrolysing the urine before extraction. Enzyme hydrolysis using extract of *Helix pomatia* (containing ß-glucuronidase and aryl sulphatase) was demonstrated to be more effective than acid hydrolysis (Section 2.2.5.3.1). The same urine dilutions and internal standards were used as for the free metabolite analyses. The hydrolysis conditions are described in Section 2.2.1.

After incubation, samples were treated identically to free metabolite samples except that, due to the acetate buffer, 40 µl 5 M HCl was required to acidify the sample. Also, the formation of emulsions in the ethyl acetate phase after vortexing required physical disruption (Section 2.2.1). Recovery of metabolites from the urine and enzyme mixture is considered in Section 2.2.5.3.2.

Table 2.3. Repeatability of GC analyses of metabolites quantified by determining the coefficient of variation (CoV) of individual calibration curves.

		Species	Coefficient of Variation % (n=5)			
		metabolites	Within day[3]		Between day	
Metabolite	n[1]	quantified in[2]	Low conc[4]	High conc[5]	Low conc[4]	High conc[5]
Cy 1	6	**R**			2.4	1.2
Cy 2	5	R, BP, RT, **GG** , K	7.3	12.7	19.4	14.9
Cy 5	5	R, BP, RT, **GG**	8.4	4.7	7.2	5.7
Cy 5 TMS[6]	2	**K**			3.1	4.9
Cy 6	5	R, BP, RT, **GG** , K	8.9	6.4	14.9	4.7
Cy 8	2	**BP**			3.0	2.3
Cy 10	3	**K**			16.4	7.9

[1]Number of calibration curves prepared. In the case of Cy 5 (TMS), 8 and 10, the number of replicates is less than required for significant CoV values, but, an indication of repeatability for those metabolites is gained.
[2]R = rat, BP = brushtail possum, RT = ringtail possum, GG = greater glider and K = koala. CoV of calibration curves are from those prepared for the species highlighted in bold.
[3]Both within-day and between-day CoVs were specifically tested for in GG calibration curve dilutions (n = 5 for each). CoV values for the other metabolites are opportunistic from calibration curves prepared throughout sample analyses.
[4,5]The low and high concentrations correspond with the lowest and highest concentration in each case.
[6]Cy 5 was analysed as both the methyl ester and methyl ester plus TMS derivative in the koala.

2.2.5.3.1. Determining the method of hydrolysis

Three replicates of enzyme and acid hydrolysis, using rat urine (after the 1.49 mmol/kg dose of *p*-cymene), were compared to control replicates.

The enzyme method used 0.5 ml urine, 50 µl internal standard (125 µg/50µl of 2,5 dimethyl benzoic acid), 0.5 ml water, 0.2 ml 1.1 M acetate buffer (pH 5.3) and 100 µl extract of *Helix pomatia.* The mixture was incubated at 37.5°C overnight

The method for acid hydrolysis combined 0.5 ml urine sample, 0.5 ml water, 50 µl of the internal standard and 40 µl 5 M HCl. The mixture was heated at 40°C overnight.

The control replicates contained only 0.5 ml urine, 0.5 ml water and the same amount of internal standard.

After their respective treatments, all replicates were acidified, extracted and analysed as previously described (section 2.2.1).

Metabolite recoveries after each treatment method are reported in Table 2.4. Enzyme hydrolysis resulted in more effective hydrolysis of all metabolites. The acid treatment failed to hydrolyse the ester glucuronide of Cy 8. The conjugate of Cy 1 was hydrolysed by both methods. The concentrations of Cy 2, 5 and 6 were not significantly increased by either method and these metabolites were therefore considered not to be conjugated. β-Glucuronidase activity is non-specific, hydrolysing both ether and ester glucuronides (extract of *Helix pomatia* product information leaflet, Boehringer Mannheim; Dutton 1980). Thus enzyme hydrolysis was chosen as the preferred method of hydrolysis.

Table 2.4. Comparison of enzyme and acid hydrolysis on metabolite recovery, compared to a control (n=3).

Hydrolysis method	Metabolite concentration µmol/ml (mean ± sd)				
	Cy 1	Cy 2	Cy 5	Cy 6	Cy8/IS[2]
Enzyme	1.39 ± 0.06	0.87 ± 0.10	0.17 ± 0.02	0.52 ± 0.09	1.10 ± 0.05
Acid	1.40 ± 0.03	0.91 ± 0.10	0.16 ± 0.07	0.42 ± 0.07	0.04 ± 0.04
Control	0.87 ± 0.08	0.92 ± 0.05	0.22 ± 0.05	0.42 ± 0.00	0.04 ± 0.01
P -value (F)[1] *df* =2 in each case	*<<0.05* (56)	*0.70* (0.4)	*0.11* (3.2)	*0.24* (1.82)	*<<0.05* (975)

[1]Anova (single factor) was used to determine the *P* -value and F statistic for the recovery of metabolites compared to the control.

[2]Cy 8 is reported as the ratio of internal standard as no calibration curve was available.

2.2.5.3.2. Metabolite recovery after enzyme hydrolysis

The addition of extract of *Helix pomatia* to urine was investigated to determine whether its presence affected the recovery of metabolites. Crude enzyme extract could potentially alter the relative partitioning of metabolites, internal standards and endogenous compounds from the urine into ethyl acetate. Furthermore, an emulsion between the two phases occurred after extracting the enzyme hydrolysate. Even after the disruption of the emulsion, an emulsion-like interface persisted between the phases. This interface could potentially act as a reservoir for metabolites. The aim of this trial was to assess the system and quantify any effect on metabolite recovery due to the presence of the enzyme extract.

Methods. Two experiments were performed to assess the recovery of metabolites from enzyme-urine mixtures. The first compared metabolite recovery from urine acidified and extracted immediately after the addition of the enzyme with a control, which was not treated with enzyme. The second experiment was identical to the first except that the enzyme had been denatured prior to adding to the urine.

For each experiment, five replicates of both the treatment and control were prepared: 0.5 ml urine (collected from rats in the 24 hr after 1.49 mmol/kg of *p*-cymene), 0.5 ml water and 50 µl internal standard solution. The enzyme treated groups had 0.2 ml 1.1 M acetate buffer and 100 µl extract of *Helix pomatia* added to each. In the second experiment, the enzyme was denatured by heating at 100°C for 2 min. All replicates were acidified and extracted immediately (ie. allowing minimal time for enzyme activity to cause hydrolysis) and analysed by GC/FID.

Metabolite and endogenous acid (hippuric and phenylacetic acid) recoveries after both treatments are compared to the control and are reported in Table 2.5.

In the first experiment, recovery of Cy 2, 5 and 6 (all shown not to undergo hydrolysis in section 2.2.5.3.1), hippuric acid and phenyl acetic acid was reduced to 85.2 ± 3.0 % (mean ± sd) of the control. Metabolite recoveries after hydrolysis were corrected by the following factors in each species: Cy 2 - 1.13, Cy 5 - 1.20 and Cy 6 - 1.13.

Table 2.5. Effect of extract of *Helix pomatia* on metabolite recovery.

	Metabolite/internal standard[1] (mean ± sd; n = 5)						
	Cy 1	Cy 2	Cy 5	Cy 6	Cy 8	HA	PA
Treatment 1							
Control	1.60 ± 0.17	2.12 ± 0.12	0.92 ± 0.07	1.03 ± 0.05	1.41 ± 0.04	2.97 ± 0.28	0.83 ± 0.08
Enzyme	3.12 ± .23	1.88 ± .12	0.77 ± .06	0.91 ± .07	1.49 ± .15	2.46 ± .26	0.69 ± .08
%	195	89	83	88	106	83	83
Treatment 2							
Control	0.87 ± 0.04	0.63 ± .03	0.17 ± .01	0.31 ± .02	-	2.90 ± .28	0.67 ± .08
Enzyme[d]	1.02 ± .13	0.64 ± .04	0.16 ± .03	0.30 ± .03	-	3.00 ± .33	0.71 ± .08
%	117	101	91	96	-	103	107

[1]Peak heights by GC/FID
Enzyme[d] - denatured by heating at 100°C for 2 min.
HA = hipurric acid and PA = phenylacetic acid.

On the other hand, Cy 1 showed a marked increase in recovery after the addition of the enzyme. The recovery of Cy 8 was also greater than that of the other metabolites and endogenous acids. Both these metabolites were shown to be hydrolysed by extract of *Helix pomatia* in Section 2.2.5.3.1. It appears hydrolysis of the Cy 1 conjugate occurred rapidly upon exposure to the enzyme extract. The second experiment supported this explanation as recovery of all metabolites was close to 100 % when the enzyme was present but its activity destroyed.

2.2.6. Urinary glucuronic acid assay

Urinary glucuronic acid concentrations were measured directly using the method of Blumenkrantz and Asboe-Hansen (1973). The method relies upon the formation of a chromogen when uronic acid is heated with conc. sulphuric acid/sodium tetraborate and treated with *m*-hydroxy diphenyl (3-phenyl phenol; Aldrich Chemical Co., Milwaukee, Wisconsin, USA). The absorbance of the chromogen is measured using spectrophotometry. The molar glucuronic acid concentration of urine could then be reconciled to the molar concentration of conjugated metabolites.

2.2.6.1. Calibration curve

A stock solution of aqueous D-glucuronic acid was prepared (200 µg/ml). Dilutions were made with distilled water to produce the following concentrations of glucuronic acid: 0, 0, 10, 20, 30, 40 µg/0.4 ml. The first dilution of 0 µg/0.4 ml was used as the blank for spectrophotometry. A linear relationship between concentration and UV absorbance occurred over this range.

The dilutions were chilled in an ice-bath before adding 2.4 ml 0.0125 M sodium tetraborate in conc. sulphuric acid. The tubes were then heated at 100°C in a boiling water bath for 5 min and then re-cooled in a water/ice bath.

To each dilution 40 µl 0.15 % *m*-hydroxy diphenyl in 0.5 % NaOH was added (freshly prepared and stored only for short periods, wrapped in foil in the refrigerator). The UV absorbance (λ 520 nm) was measured after adding and mixing

the *m*-hydroxy diphenyl. A Cecil Instruments CE20 ultraviolet spectrophotometer was used to measure the absorbance of all samples, except for koala samples which were measured using a Varian Cary 1E UV-Visible Spectrophotometer (Varian Australia Pty, Ltd, Mulgrave, Victoria).

Each day samples were assayed a new calibration curve was prepared. A total of 14 calibration curves were prepared throughout this study and the mean calibration equation was:

$$y = 0.036x - 0.012 \qquad R^2 = 0.995$$

The coefficients of variation for a low (10 µg/0.4ml) and high (40 µg/0.4ml) concentration and calibration curve slopes were 16.6, 6.3 and 7.0 % respectively.

2.2.6.2. Assay of urine samples

The same method as described above was used for the assay of all urine samples. D-glucuronic acid dilutions were substituted for diluted urine. Appropriate urine dilutions are reported for each species in their respective chapters.

2.2.7. Analysis of faeces for *p*-cymene metabolites

A sample of faeces from each species was reduced to a slurry by adding approximately eight times its weight of water and allowing the mixture to stand at room temperature overnight. The samples were shaken periodically to assist disintegration of pellets. The final volume of faecal slurry was weighed and a portion analysed for free and total *p*-cymene metabolites in a similar manner to urine samples.

Five ml aliquots of slurry were placed into large (30 ml) centrifuge tubes along with internal standard (62.5µg 2,5 dimethyl benzoic acid in 25 µl methanol). Two ml of 1.1 M acetate buffer (pH 5.3) and 200 µl extract of *Helix pomatia* was added and the mixture left to incubate overnight at 37.5°C. The samples were acidified with 200 µl 5 M HCl and then extracted with three washes of ethyl acetate (1 x 9 ml then 2 x 6 ml). The tubes were mixed on a vortex mixer for 1 min and then centrifuged at 2000 rpm for 5 min to separate the phases. Ethyl acetate was evaporated on a rotary evaporator to concentrate the sample before methylating approximately 0.5 ml of the concentrated extract with 1.5 ml of diazomethane using conditions described previously (Section 2.2.1). Excess diazomethane was evaporated with a stream of nitrogen.

Samples were then analysed by gas chromatography using the conditions described in Section 2.2.2.

Table 2.6. Mass spectra and retention times for *p* -cymene metabolites identified. Mass spectra are for derivatised metabolites (Table 2.1).

Metabolite	Rt[1] (min)	Significant EI ions (*m/z*) — *Relative abundance (%)*															
Cy 1	3.55	150	136	135	132	119	117	115	91	89	77	67	65	63	51	44	43
		6	*6*	*49*	*30*	*3*	*4*	*4*	*14*	*3*	*4*	*3*	*8*	*5*	*4*	*3*	*100*
Cy 2	9.48	194	179	163	137	135	105	91	77	59	43						
		2	*100*	*11*	*16*	*7*	*13*	*12*	*14*	*16*	*78*						
Cy 3	10.51	194	136	135	117	115	107	105	103	91	79	77	44				
		26	*11*	*100*	*9*	*5*	*12*	*40*	*10*	*15*	*22*	*20*	*12*				
Cy 4	4.70	150	120	119	117	115	104	91	77	65	44						
		17	*13*	*100*	*17*	*8*	*6*	*28*	*10*	*7*	*4*						
Cy 5	11.71	194	163	149	131	117	105	91	77	59	51						
		14	*100*	*12*	*22*	*11*	*29*	*33*	*27*	*25*	*10*						
Cy 6	11.87	222	191	163	131	104	103	91	77	59	51						
		30	*14*	*100*	*16*	*14*	*20*	*15*	*15*	*18*	*6*						
Cy 8	6.35	178	163	147	131	119	117	104	103	91	78	77	59	51	50	41	
		48	*100*	*40*	*25*	*58*	*13*	*17*	*27*	*59*	*15*	*32*	*29*	*20*	*10*	*20*	
Cy 9	5.31	178	120	119	117	103	91	77									
		20	*11*	*100*	*12*	*5*	*19*	*7*									
Cy 10	16.54	235	147	131	104	103	91	77									
		19	*100*	*6*	*7*	*7*	*12*	*9*									
Cy 11	16.88	233	201	145	117	116	115	102	91								
		2	*100*	*11*	*16*	*7*	*13*	*12*	*14*	*16*	*78*						
Cy 12[2]	-	310	295	133	131	115											
		0	*100*	*14*	*30*	*4*											
Cy 13	-	282	252	251	179	161	148	137	120	105	103	91	77	75	73	59	43
		tr	*18*	*9*	*100*	*24*	*16*	*9*	*8*	*8*	*9*	*5*	*8*	*23*	*36*	*7*	*41*

[1]Rt (retention times) were measured by GC/FID. See Section 2.2.2 for conditions. Cy 13 was detected using GC/MS and therefore the retention times were not comparable to the others. Cy 13 eluted later than all other non-conjugated metabolites.

[2]Cy 12 was also identified by GC/MS as its TMS derivative and was not analysed by GC/FID.

2.3. Results

The chemical structures of all *p*-cymene metabolites identified are shown in Figure 2.1 and the names assigned to these metabolites for the purpose of this study, plus their chemical names, source of mass spectra data, whether they were quantified, the derivatisation required and the purity of standards used for quantitation are shown in Table 2.1. The GC/FID retention times, relative abundance of molecular ions (M^+) and the major mass spectral fragments of the metabolites are listed in Table 2.6.

p-Cymene metabolites were initially identified by direct comparison of GC pre- and post-dose urine extracts (Figure 2.2). All chromatogram peaks occurring in the post-dose urine but not the pre-dose urine were considered to be *p*-cymene derived and therefore investigated. Mass spectra of each peak were compared to published data, which successfully identified all but one metabolite, Cy 13. Identification of this metabolite is detailed in Chapter 7 - section 7.2.5 and 7.3.2.

The extent of metabolite oxidation was clarified by grouping metabolites according to the number of oxygen atoms acquired during oxidation. For this purpose, Cy 1 was the sole quantifiable metabolite acquiring only one oxygen (Cy 4 was detected as only a trace in the rat and brushtail possum and Cy 7 was not detected at all), Cy 8, 9 and 10 all acquired two oxygens (trace amounts of Cy 12, a dihydroxy cymene compound, was detected in the rat only and not quantified). Cy 10 (cuminuric acid), a glycine conjugate of Cy 8 was included in this group as it acquired two oxygens prior to conjugation. Cy 2, 3 and 5 all acquired three oxygens and Cy 6 and 13 acquired 4 oxygens each.

Urinary glucuronic acid assay measurements using the colorimetric method described were often variable. Therefore limited significance could be attached to the results obtained, however, where appropriate they have been included as they provide an indication, at least, of the urinary excretion of glucuronic acid. Glucuronic acid assays reported in Part 2 of this thesis were less variable as UV readings measured 5 min after the addition of the *m*-hydroxy diphenol were more stable than those measured within 5 min, as instructed in the method of Blumenkrantz and Asboe-Hansen (1973).

Analysis of faecal extracts detected no *p*-cymene metabolites and only trace amounts of unchanged *p*-cymene in any of the species studied.

2.4. Discussion

The ability to match mass spectra with the contents of the customised and commercial mass spectra libraries provided a powerful tool for identifying the metabolites of *p*-cymene. Fortunately, the metabolism of *p*-cymene had been studied and reported in other species and all metabolites, apart from Cy 13, identified in this study had published mass spectra. Identification of metabolites based on mass spectra alone was suitable for the purpose of this study, especially as the mass spectrometry of the majority of *p*-cymene metabolites produced molecular ions. Walde *et al.* (1983) reported a total of 16 *p*-cymene metabolites in rat urine, of which nine were present in minor amounts (< 4%, or as trace amounts only). The five

major metabolites identified in our study corresponded to the five predominant metabolites reported by Walde, although there were differences in relative abundances. Despite extracting mass chromatograms of diagnostic ions from theGC/MS TIC, seven of Walde's trace metabolites were not detected in this study. It may be that they were present below the detection limits of this assay due to the small volume of urine sampled. Walde's assay used large urine volumes, which were first fractionated to yield acidic and neutral fractions. Furthermore, Walde used on-column GC/MS injection of urine extracts, resulting in improved sensitivity in the chromatography. The unidentified metabolites were four diols, a glycine conjugate, its immediate precursor, and a tertiary alcohol, cuminyl alcohol (Cy 7).

Mass spectrometry of *p*-cymene metabolites generally produced M^+ ions. All metabolites fragmented to form large *m/z* ions after a β cleavage to the aromatic ring. For many metabolites (Cy 2, 3, 4, 5, 6, 8, 9, 10 and 12) this was the base ion. The fragmentation pattern of the methyl ester and TMS derivative of Cy 13 is examined in Chapter 7 - Figure 7.5.

The model carboxylic acids used to develop the saponification method were chosen for their relative similarities to metabolites. The experiments were successful in that they confirmed that methylated carboxylic acids saponify under simple conditions. However, the time required to saponify the model carboxylic acids, especially 2,5-dimethyl benzoic acid (methyl ester), was much greater than that taken by metabolites. In retrospect, it is apparent that the methyl group adjacent to the carboxylic acid moiety in 2,5 dimethylbenzoic acid caused steric hindrance protecting the methyl ester from hydrolysis and therefore slowing the saponification reaction.

In comparison, saponification of metabolites occurred rapidly (within 4 h). The reaction was successfully followed by sampling the control reaction mixture at frequent intervals.

The chemical stabilities of ether and ester glucuronides are markedly different (Dutton 1980). Ether glucuronides tend to require treatment with hot concentrated acid for hydrolysis whereas ester glucuronides are generally hydrolysed by warm dilute acid. β-Glucuronidase, however, is non-specific and, as was demonstrated in Section 2.2.5.3.1, hydrolyses both types of glucuronides. The conditions employed to hydrolyse urine samples in this study were expected to hydrolyse ester glucuronides and it is therefore interesting to note that the ester glucuronide of Cy 8 did not hydrolyse. Aggressive acid treatment required for hydrolysing ether glucuronides was unsuitable for urine samples as the stability and volatility of metabolites under these conditions was unknown.

Metabolite recovery from urine was shown to be reduced by extract of *Helix pomatia*. Metabolite recovery after hydrolysis was 83 - 89 % of unhydrolysed urine (ie. approximately 15 % of extractable metabolites were not recovered). This value was consistent for hippuric acid, phenyl acetic acid and *p*-cymene metabolites Cy 2, 5 and 6. The mode by which the enzyme inhibited metabolite recovery is speculative. Its effect may be associated with the surfactant properties of the system which also results in the formation of an emulsion between the urine and solvent., or it may be associated with excess salts in the crude extract. Cy 1 and 8, on the other hand, had increased recoveries, indicating that extensive hydrolysis had occurred before the extraction was complete. It seems the metabolite conjugates

hydrolyse very rapidly in the presence of β-glucuronidase, as the extraction process started immediately upon the addition of the enzyme. Recovery of all metabolites and endogenous compounds approached 100 % with the addition of the denatured enzyme. This confirms that enzyme activity was the cause of increased recovery of Cy 1 and 8.

Inactivation of enzymatic activity by heating at 100°C for 10 min caused the extract to solidify, rendering it impossible to work with. The extract used was exposed to heat for only 2 min, by which time it was semi solid, but still measurable. Remnant activity in the enzyme extract may have caused a small amount of Cy 1 hydrolysis in the second experiment, as its recovery was 117 % (a significant reduction from 195 % after the first trial).

Quantified metabolite recoveries after hydrolysis were adjusted by the correction factors measured in the first experiment. The average of the values measured for Cy 2, 5, 6, hippuric acid and phenylacetic acid was applied to Cy 1 and 8 for which no direct measurement was obtained.

The absence of unchanged *p*-cymene in the faecal extracts was not surprising. The lipophilic nature of *p*-cymene ensures it is rapidly absorbed from the gastrointestinal tract (Foley *et al.* 1987). The molecular weights of oxidised metabolites (150 - 194) are below the threshold for biliary excretion (350 - 400 amu; Pratt and Taylor 1990), although the molecular weights of the glucuronide conjugates are approaching the weight required for elimination. For example, the glucuronide of another terpene, menthol, has been reported to form menthol glucuronide which undergoes extensive biliary excretion and enteroheptaic circulation in the rat (Yamaguchi *et al.* 1994).

CHAPTER 3

P-CYMENE METABOLISM IN THE RAT

3.1. Introduction

The metabolism of *p*-cymene in the laboratory rat was investigated as a preliminary to further studies on its metabolism in marsupial folivores. The rat was included in the comparison as its metabolism of *p*-cymene has previously been reported in detail (Walde *et al.* 1983). It therefore provides a eutherian standard by which the metabolism of *p*-cymene in marsupials can be compared. Experimental techniques were developed using urine and faeces collected from the rat and these are detailed in Chapter 2. Methods specific to the analysis of rat urine, as well as qualitative and quantitative results from urine analyses, are described in this chapter.

3.2. Methods

3.2.1. Animals and Dosing

Six hooded wistar rats (weight 274 ± 59 g (mean ± sd); 4 females and 2 males; identified as R 1 - 6) were obtained from stocks held in the University of Tasmania. The rats were administered two single oral doses of *p*-cymene (0.37 and 1.49 mmol/kg) in peanut oil. Doses were administered at least two weeks apart to ensure no carryover effect of the first dose on metabolism enzyme activity. Two separate *p*-cymene mixtures were prepared (0.06 and 0.30 mmol/ml) to provide dose volumes of less than 2 ml. The doses were administered by oral gavage using a curved, blunt needle (18 G x 5 cm), with a rounded bulb soldered to the tip.

The rats were placed in round glass metabolism cages (25 x 18 cm; diamter x height). The floor of the cages was mesh which allowed urine and faeces to drop through. Urine was directed onto the sides of the collecting funnel and flowed into a side collecting vessel. Faeces were separated from urine in the collecting funnel, dropping straight into a collecting vessel. Both faeces and urine collecting containers were kept in ice during the collecting periods. Pre-dose samples (day 0) were collected for 24 h before the *p*-cymene doses were administered. Two 24 h samples of urine and faeces were collected post-dose (days 1 and 2).

The rats were housed in a light (12 h light/dark cycle) and temperature (20°C) controlled room. They were fed on a diet of standard rat chow pellets (University of Tasmania, Animal House) and water was provided *ad lib.*

The walls and floors of cages were washed at the end of each collection period with a minimal volume of distilled water to remove residual urine. The washings

were added to the urine and the total volume used in the analysis of urine. Metabolite recovery was improved by this procedure.

Urine volume and pH were noted and faeces were also collected and weighed. Both urine and faeces were stored at -18°C until analysed.

All rats were euthenased with carbon dioxide at the end of the experiment.

3.2.2. Urine analyses

Free and total urinary metabolites were analysed in rat urine collected after each dose of *p*-cymene. One ml of undiluted urine was used for the 0.37 mmol/kg dose while urine was diluted (1:2) for the 1.49 mmol/kg dose. The assay for free and total metabolites is described in Chapter 2 - sections 2.2.5.2 and 2.2.5.3, respectively.

3.2.3. Quantitation of urinary metabolites

Metabolites were identified by their GC retention times and mass spectra as described in Chapter 2 - section 2.2.3 and Table 2.6. The major metabolites, Cy 1, 2, 5, 6 and 8 were all quantified (see Figure 3.1. and Chapter 2 -Table 2.1 for chemical structures and names of metabolites). Isolation of Cy 1, 2, 5 and 6 for calibration standards used pTLC for the preliminary separation and clean up. Final purification of crudely separated metabolites used pHPLC as described in Chapter 2 - section 2.2.4.

The calibration curve prepared for Cy 2 was used to quantify Cy 8. This was because Cy 8 was only detected upon hydrolysis of real urine samples, after calibration curves had been prepared. Since Cy 2 and 8 have the same number of carbon atoms, they would be expected to have similar response factors by GC/FID (Jorgensen *et al.* 1990). This was shown to be the case in a later experiment when Cy 8 (cumic acid; purchased commercially) was quantified in brushtail possum urine (Chapter 4). The calibration curve slopes of Cy 2 and 8 were comparable (0.801 and 0.804, respectively; Appendix 3 - Table A3.1).

The purities of isolated Cy 1, 2, 5 and 6 were, by GC/FID, 92.7, 97.1, 93.2 and 99.4 % respectively. Impurities in the isolated metabolites did not interfere with metabolite peaks and calibration curve dilutions were adjusted for weight to compensate for impurities. Calibration curves were prepared for Cy 2, 5, and 6 as described in Chapter 2 - section 2.2.5.1 and details of the calibration curves are reported in Appendix 2 - Table A2.1.

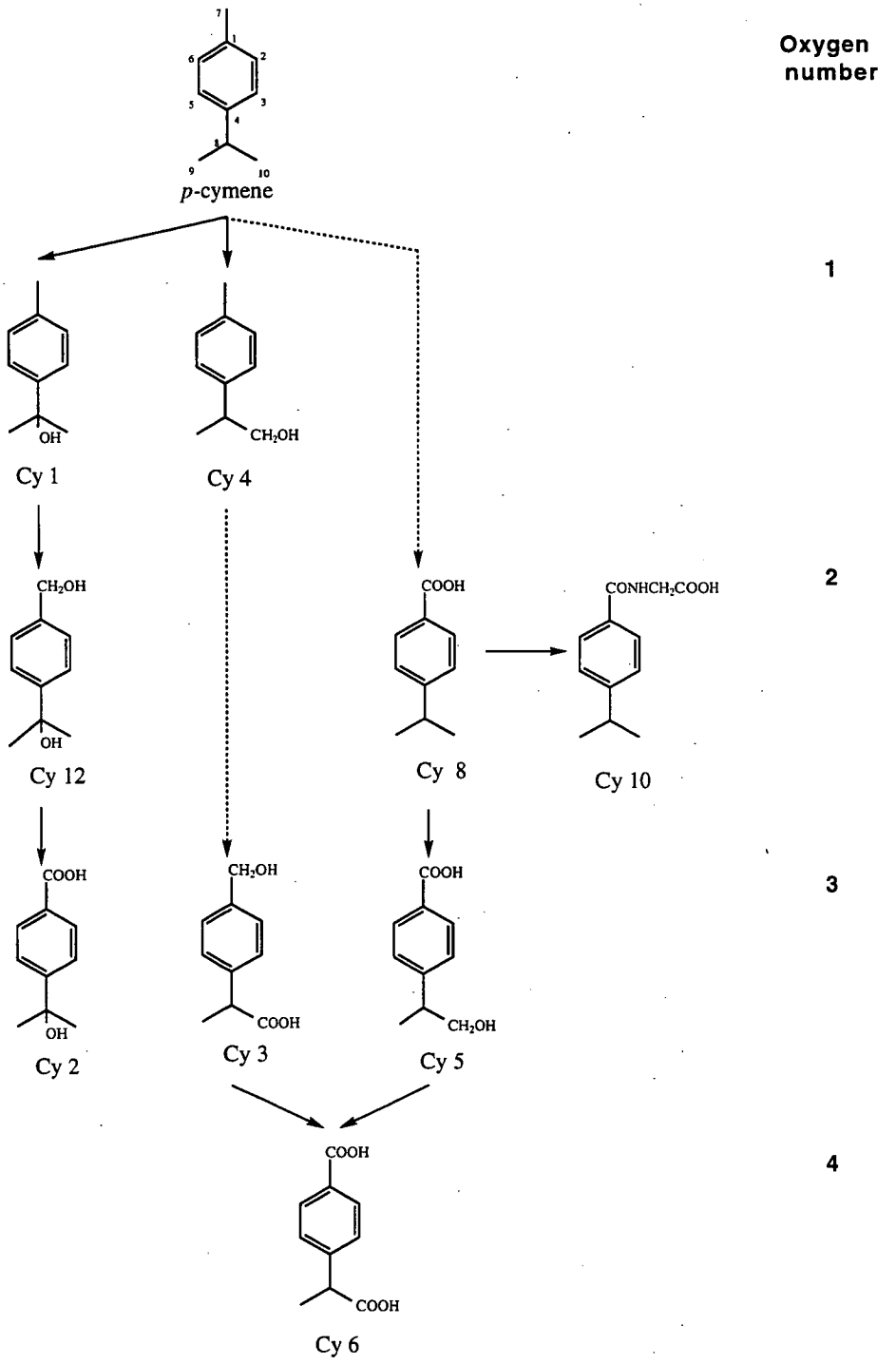


Figure 3.1. Chemical structures and proposed metabolic pathways of *p* -cymene metabolites in the rat. Metabolites are shown in their underivatised forms. Dashed arrows indicate where intermediate metabolites have not been detected. Number of oxygen atoms acquired during oxidation are reported on the right hand side of the diagram. See Chapter 2 - Table 2.1 for chemical names.

3.3. Results

3.3.1. Qualitative results

Nine metabolites, Cy 1, 2, 3, 4, 5, 6, 8, 10 and 12, were identified in the urine of the rat (Figure 3.1 and Chapter 2 - Table 2.1 for chemical structures and names). Cy 3, 4, 10 and 12 were detected as trace metabolites.

3.3.2. Quantitative results

A typical gas chromatogram of pre- and post-dose urine extracts after hydrolysis was shown in Chapter 2 - Figure 2.2. The pre-dose urine extract had only a few peaks, whereas the post-dose urine had multiple peaks derived from *p*-cymene.

p-Cymene metabolites were quantified in urine from six rats after 0.37 and 1.49 mmol/kg of *p*-cymene. Fractional recovery of administered doses were 0.65 ± 0.12 (mean ± sd; range 0.43 - 0.75) and 0.52 ± 0.09 (mean ± sd; range 0.40 - 0.63) for the low and high doses of *p*-cymene, respectively. Most was recovered in the first 24 h after the *p*-cymene dose: after 0.37 mmol/kg of *p*-cymene, 98 ± 3 % (mean ± sd) and after 1.49 mmol/kg of *p*-cymene, 96 ± 5 %. Although samples were stored and analysed separately, results for both post-dose days were combined for the purpose of studying the metabolic pattern of excretion.

Figure 3.2 depicts pictorially the pattern of metabolite excretion and reports the molar recovery of each metabolite per body weight (kg^{-1}). Hydrolysis increased the recovery of Cy 1 and 8 (Figure 3.2).

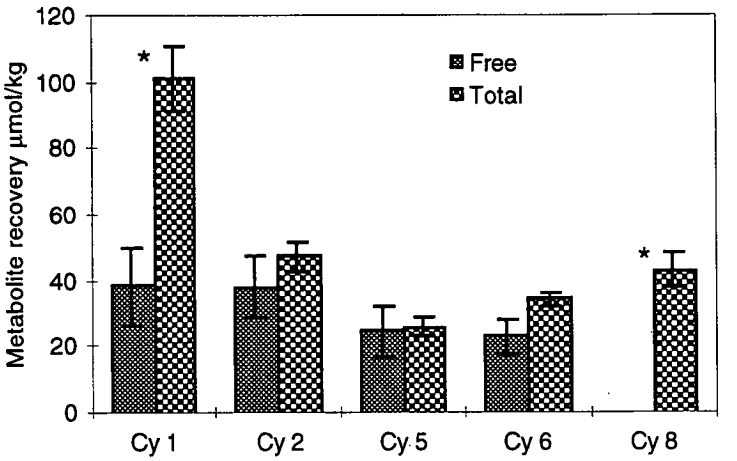
To minimise the effect of variable recoveries of administered dose between individual rats, metabolite recoveries were standardised as the percentage total of recovered metabolites. This allowed a direct comparison of the pattern of metabolite excretion between rats and reduced the variability in the results. The excretion of free, conjugated and total metabolites is reported in Table 3.1. Cy 1, a simple hydroxy metabolite was the most abundant metabolite, accounting for 35 - 40 % of total metabolites after each dose. Cy 2, a more extensively oxidised metabolite, accounted for 19 - 22 % of the metabolites. The most oxidised metabolite, Cy 6, accounted for less than 15 % of the total metabolites (Table 3.1).

Two rats showed minimal conjugation of any metabolites after the 0.37 mmol/kg dose of *p*-cymene which resulted in large standard deviations for that dose, making statistical comparison between the two doses insensitive. It is unclear why these two rats exhibited this pattern of conjugation. After the higher dose of *p*-cymene, the same rats had comparable patterns of conjugation to the other rats.

There was a marked drop in the percentage of total metabolites excreted in the conjugated form after the higher dose (47 % after 0.37 mmol/kg compared to only 25 % after 1.49 mmol/kg). Approximately 60 % of Cy 1, the least oxidised metabolite, was conjugated after 0.37 mmol/kg whereas this had dropped to only

23 % after 1.49 mmol/kg *p*-cymene. Cy 8 was essentially completely conjugated after both doses. Cy 2, 5 and 6 showed minimal conjugation after either dose.

A) Dose 0.37 mmol/kg



B) Dose 1.49 mmol/kg

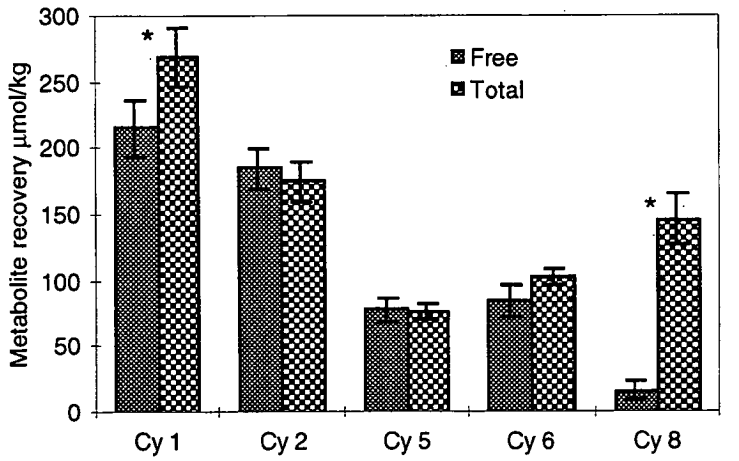


Figure 3.2. Molar recoveries (µmol/kg; mean ± se) of free and total levels for each metabolite excreted by the rat after *p* -cymene. A) 0.37 mmol/kg (n = 6) and B) 1.49 mmol/kg (n = 5). Metabolite recovery was significantly increased by hydrolysis for those metabolites marked with * (Student's paired *t* -test, $P < 0.05$).

Table 3.1. Excretion of free, conjugated and total metabolites expressed as a percentage of total recovered metabolites after both doses of *p* -cymene in the rat (n = 6).

	Percent (mean ± sd) of total urinary metabolites					
	Dose 0.37 mmol/kg			Dose 1.49 mmol/kg		
Metabolite	Free	Conjugated	Total	Free	Conjugated	Total
Cy 1	15.9 ± 3.9	23.8* ± 11.8	39.7 ± 2.5	28.0 ± 5.1	8.2* ± 4.2	35.0 ± 3.6
Cy 2	16.9 ± 11.6	5.9 ± 4.7	18.9 ± 2.6	24.3 ± 5.3	0.9 ± 1.4	22.6 ± 1.1
Cy 5	11.8 ± 12.1	3.5 ± 3.9	10.6 ± 2.7	10.3 ± 3.1	0.5 ± 0.4	10.1 ± 2.0
Cy 6	10.5 ± 8.0	4.9 ± 4.9	14.0 ± 2.7	11.3 ± 4.7	3.0 ± 1.8	13.4 ± 1.2
Cy 8	0.0 ± 0.0	16.8 ± 2.4	16.8 ± 2.4	2.3 ± 2.1	15.9 ± 4.4	18.9 ± 3.5
Sum	55.1† ± 39.8	46.9‡ ± 36.7	100†	76.2† ± 17.2	25.2‡ ± 14.3	100†

*Comparison of percentages between doses, $P < 0.05$ (Student's paired *t* -test).
†Comparison of free and total for each dose, $P < 0.05$ (Students paired *t* -test).
‡There was no significant difference in the percent of glucuronides between the doses (Students *t* -test (paired) P>0.05).
Each value is the mean ± sd of individual rats. Free and total metabolites were determined, respectively, before and after hydrolysis, and the difference was considered to be due to the conjugated metabolites. For some metabolites free levels were slightly greater than total, and the value of the difference was considered to be "0".
Fractional recoveries of *p* -cymene were 0.65 ± 0.12 and 0.52 ± 0.09 for the low and high dose respectively.

Metabolites were grouped according to the number of oxygen atoms acquired during oxidation (Figure 3.1). Cy 1 (one oxygen) accounted for the largest portion of the recovered dose, and Cy1 and Cy 8 (two oxygens) together accounted for more than half of the recovered metabolites (Figure 3.3).

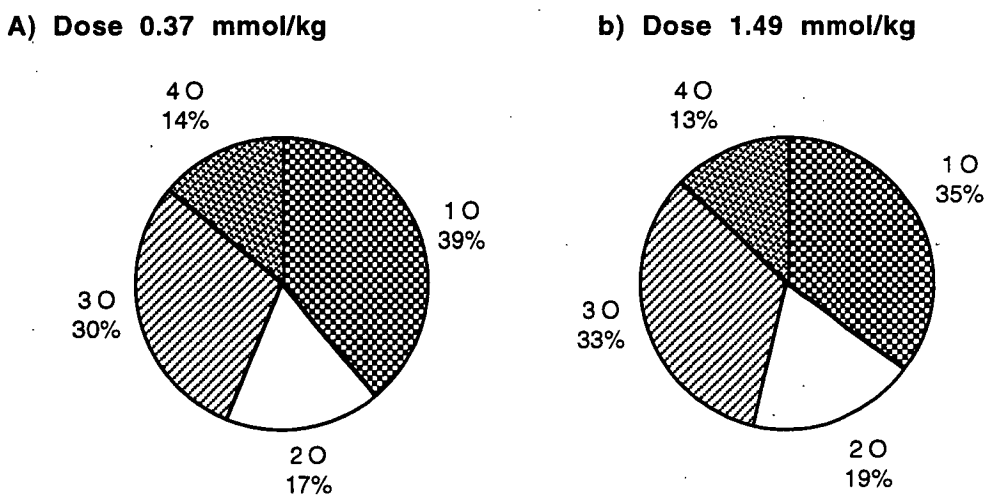


Figure 3.3. Metabolites grouped according to the number of oxygen atoms (O, 1 to 4) acquired during oxidation and reported as the percent of recovered dose, after hydrolysi.

3.3.3. Glucuronic acid excretion

Glucuronic acid was found in the urine of all rats, including significant amounts in the pre-dose samples (159 ± 80 and 185 ± 51 μmol/kg (mean ± sd) for 0.37 and 1.49 mmol/kg *p*-cymene, respectively). There seemed to be variability in the results returned by the colorimetric glucuronic acid assay so that reconciliation of calculated conjugated metabolite levels and glucuronic acid measurements was only moderately successful (Appendix 2 - Table A2.3). Increases in glucuronic acid (adjusted for pre-dose output) were comparable to the molar concentration of conjugated metabolites after the lower dose, however, after the higher dose, the glucuronic acid exceeded the conjugated metabolites. Glucuronic acid concentrations returned to pre-dose levels by day 2 (the second post-dose day). There correlation between post-dose molar glucuronic acid amounts and molar metabolites conjugated, was however, reasonable ($R^2 = 0.67$, $P < 0.001$, $df = 19$).

Glucuronic acid levels were not measured in two rats after the 0.37 mmol/kg dose as all available urine had inadvertently been used for metabolite harvesting.

3.3.4. Animal details

Individual rat details including weight, *p*-cymene dose, molar recoveries of metabolites and urine volumes and pH are recorded in Appendix 2 - Tables A2.2 and A2.4. pH measurements of fresh pre-dose urine were neutral (pH 6.8 ± 0.2). There was little change in urine pH after the dose. Urine output was reasonably consistent during the experiments (17.5 ± 4.3 ml per day throughout the 0.37 mmol/kg dose and 16.4 ± 6.9 ml throughout the 1.49 mmol/kg dose).

3.4. Discussion

The metabolic fate of *p*-cymene in the rat is complex. A total of nine *p*-cymene metabolites were identified in the urine and five were present in amounts sufficient to quantify. All the metabolites underwent oxidation before excretion and they encompassed a range of oxidation states (one to four oxygens having been acquired), with the lesser oxidised metabolites (one and two oxygens) accounting for the majority of the recovered metabolites.

The pattern of metabolite excretion reported in this study was comparable to that reported by Walde *et al.* (1983) who administered an oral dose of 100 mg/kg. Although the major metabolites reported in each study were the same, there were differences in the abundances of each. In this study, Cy 1 was the most abundant metabolite observed (35 - 39 %). (Walde *et al.* 1983)Walde, however, found that the more oxidised metabolites, Cy 8 and 6, were the major metabolites after hydrolysis (19 % and 16 % respectively) and Cy 1 accounted for only about 10 % of the recovered dose. Furthermore, (Walde *et al.* 1983)Walde accounted for 80 % of the dose, significantly more than the recovery reported here (65 % and 52 % for the 0.37 and 1.49 mmol/kg doses respectively). Different methods of extraction and quantitation were used which could account for some of the differences (discussed in Chapter 2 - section 2.4).

The fact that the major metabolite excreted by the rat in this study was the least oxidised suggests that the rat is inefficient at oxidising *p*-cymene. The rat does have some ability to produce more extensively oxidised metabolites, as shown by the production of Cy 6. Glucuronidation of Cy 1, however, also has the effect of increasing polarity and therefore renal excretion. Interestingly, the rat kidney is able to excrete substantial amounts of the relatively non-polar, unconjugated Cy 1. Yet Cy 8, also a relatively non-polar metabolite, was excreted exclusively as its glucuronide. The more polar metabolites (Cy 2, 5 and 6) were excreted predominantly in their free forms.

The proportion of conjugated metabolites was less after the higher *p*-cymene dose suggesting dose-dependent glucuronidation. Yet, molar urinary glucuronic acid measurements greatly exceeded the molar conjugated metabolites. The cause of this disparity is difficult to explain. The stability of glucuronide metabolites was considered. Cy 1 forms an ether glucuronide, which is stable, and therefore unlikely to be hydrolysed in normal urinary conditions (Dutton 1980). However, glucuronide esters are susceptible to hydrolysis, particularly in warm, acidic conditions (Dutton 1980). Cy 8 forms an ester glucuronide, but there is no evidence of hydrolysis, as it was only found as the glucuronide. Urine was collected and stored in such a way to minimise the likelihood of hydrolysis. Furthermore, this disparity was not seen in the other dose or in results from other species.

Trace amounts of cuminuric acid (Cy 10), the glycine conjugate of Cy 8, were detected. The glycine conjugation pathway is active in the rat, as the glycine conjugate of benzoic acid, hippuric acid, was excreted, yet this pathway was not involved to any significant extent in the detoxification of *p*-cymene.

The overall pattern of metabolite excretion between the two doses varied only in the relative abundance of Cy 2. The similarities between the doses in the metabolites excreted suggests that there was minimal or no saturation of oxidative pathways across the dosage range administered.

Oxidation occurred on both the methyl and isopropyl groups of *p*-cymene. No ring oxidation was evident, although Walde *et al.* (1983) reported that the guinea pig excreted minor amounts of phenolic metabolites.

Recovery of the *p*-cymene dose was relatively low, especially after the higher dose of *p*-cymene. General consideration of recovery is given in Chapter 8 - section 8.3.4. However, in the case of the rat dosing experiments, techniques and familiarity with the methods were being developed, and this may have impacted on the quality of the results.

CHAPTER 4

P-CYMENE METABOLISM IN THE BRUSHTAIL POSSUM

4.1. Introduction

This chapter reports on a study of the metabolism of *p*-cymene in the common brushtail possum (*Trichosurus vulpecula*).

4.2. Methods

4.2.1. Animals and Dosing

Six brushtail possums, (weight 3.07 ± 0.84 kg (mean ± sd); 4 females and 2 males; identified as BP 1 - 6) were caught in treadle traps in bushland around the Hobart area. BP-5 had a pouch young, which emerged during the experimental period. The young was kept with its mother throughout.

The possums were housed in large enclosures (2.5 x 4 x 2.5 m) at the University of Tasmania's Animal House. The enclosures were furnished with aerial branches and nest boxes. Possums were fed a variety of food including standard rabbit pellets, bread, fruit and vegetables (carrots, apples, cabbage and banana). Possums were allowed to acclimatise to captivity for three weeks before commencing dosing experiments.

Possums were transferred to metabolism cages (39 x 45 x 38 cm) for the dosing experiments and kept in a light and temperature controlled room maintained at 20°C and a 12 h day/night light cycle. The possums were maintained on the same terpene free diet and provided with honey in water (5 %).

Possums were administered two single oral doses of *p*-cymene (0.37 or 1.49 mmol/kg) in peanut oil. Two separate *p*-cymene mixtures were prepared (0.15 and 1.05 mmol/ml) to provide a dose volume of less than 10 ml. The second dose was administered approximately three weeks after the first. The doses were administered by oral gavage using a flexible Gentle-Feed paediatric feeding tube (8.0 Fg x 38 cm, Mallinckrodt Medical, Ireland). Possums were restrained in cloth bags and held securely while a dowel gag was placed in the open mouth. A small amount of honey applied to the gag was inevitably licked by the possum, providing an opportunity to position the gag between the teeth. The feeding tube was threaded through a hole in the gag, centred above the oesophagus, and inserted into the stomach. The dose was then injected directly into the stomach. This method of administration was chosen to ensure a known dose was ingested by the possums.

Possums were returned to their metabolism cages. Urine and faeces were separated by a mesh grate placed over the urine collecting funnel. Urine drained into collecting

vessels positioned in a thermos flask containing crushed ice. The floor, walls and collecting funnel of each cage were washed with distilled water after each collection period to remove residual urine. The washings were collected and combined with the urine after urine volume and pH had been noted. The combined urine and washings were used in the analyses of the urine. Urine and faeces were collected for two 24 h post-dose periods (days 1 and 2). An 8 h pre-dose sample was collected from each possum (day 0). Possums were provided with food and water *ad lib.* Food scraps were separated from the faeces. Both urine and faeces were stored at -18°C.

All brushtail possums were returned to their point of capture at the end of the experiments.

4.2.2. Urine analyses

Metabolites were identified by their GC retention times and mass spectra as described in Chapter 2 - section 2.2.3 and Table 2.6. Free and total urinary metabolites were analysed in the brushtail possum urine collected after each dose of *p*-cymene. Undiluted urine was used for the 0.37 mmol/kg dose while urine was diluted (1:2) for the 1.49 mmol/kg dose. The assay is described in detail in Chapter 2 - sections 2.2.5.2 and 2.2.5.3.

4.2.3. Quantitation of urinary metabolites

The major metabolites (Cy 2, 5, 6, 8 and 9) were all quantified (see Figure 4.1. and Chapter 2 -Table 2.1 for chemical structures and names of metabolites). pTLC was used for the preliminary separation and clean up of Cy 2, 5 and 6. Further purification of crudely separated metabolites used pHPLC as described in Chapter 2 - section 2.2.4. Cy 8 (cumic acid) was purchased commercially (see Chapter 2 - section 2.2.4). Cy 9 proved difficult to isolate and was quantified using the Cy 8 calibration curve since it is a structural isomer of Cy 8.

The purities of isolated Cy 2, 5, 6 and purchased Cy 8 were 92, 100, 93 and 99 %, respectively. Impurities in the isolated metabolites did not interfere with metabolite peaks and calibration curve dilutions were adjusted for weight to account for impurities. Calibration curves were prepared for Cy 2, 5, 6 and 8 as described in Chapter 2 - section 2.2.5.1 and details of the calibration curves are reported in Appendix 3 - Table A3.1. Cumic acid (Cy 8) was not derivatised and was therefore added to calibration dilutions after the hydrolysis of the other isolated methylated metabolites (the same method was used for incorporating Cy 1 into the rat calibration curve dilutions described in Chapter 2 - section 2.2.5.1.4).

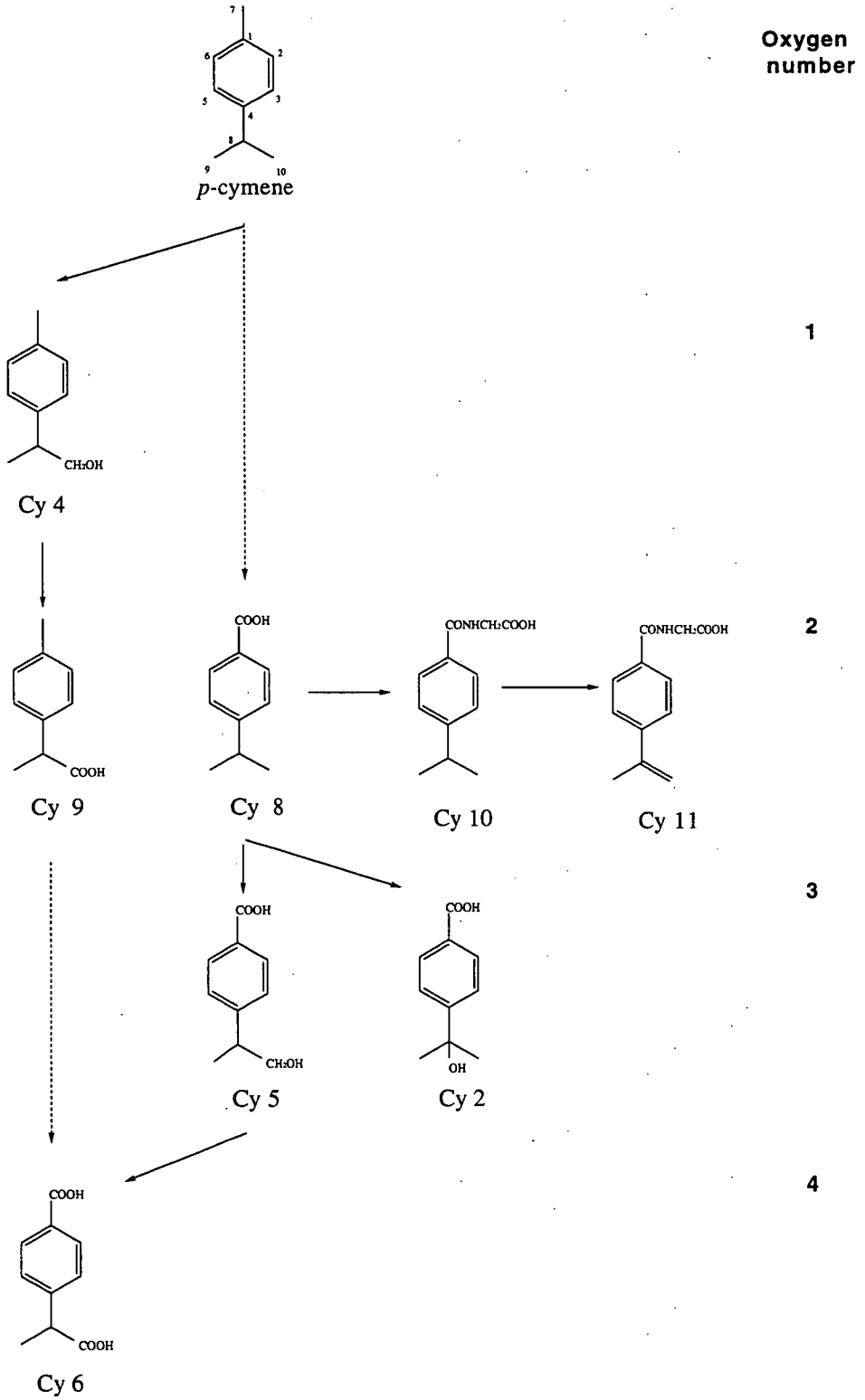


Figure 4.1. Chemical structures and proposed metabolic pathways of *p* -cymene metabolites in the brushtail possum. Metabolites are shown in their underivatised forms. Dashed arrows indicate where precursor metabolites have not been detected. See Chapter 2 - Table 2.1 for chemical names. Number of oxygen atoms acquired during oxidation are reported on the right hand side of the diagram.

4.3. Results

4.3.1. Qualitative results

Figure 4.2 shows a typical gas chromatogram of pre- and post-dose urine extracts after hydrolysis. The pre-dose urine extract has only a few peaks, whereas the post-dose urine has multiple peaks derived from *p*-cymene.

Eight metabolites (Cy 2, 4, 5, 6, 8, 9, 10 and 11) were identified in the urine of the brushtail possum (Figure 4.2 and Chapter 2 - Table 2.1 for chemical structures and names).

Cy 2, 5, 6 and 8 were familiar metabolites, having been identified as significant metabolites in other species reported in this study. Cy 4, 10 and 11 were also detected as trace metabolites in the rat. Cy 9, however, was detected only in the brushtail possum, although Walde *et al.* (1983) reported it in the rat urine as a trace metabolite.

4.3.2. Quantitative results

p-Cymene metabolites were quantified in urine from six possums after 0.37 and 1.49 mmol/kg of *p*-cymene. Fractional recoveries of administered doses were 0.62 ± 0.17 (mean ± sd; range 0.48 - 0.87) and 0.60 ± 0.14 (mean ± sd; range 0.43 - 0.79) for the low and high doses of *p*-cymene, respectively. The percentage of the dose recovered in the first 24 h was variable, generally reflecting urinary output. After 0.37 mmol/kg of *p*-cymene, 15 % (median; range: 0 - 81 %) was excreted in the first 24 h and after 1.49 mmol/kg of *p*-cymene, 88 % (median; range: 12 - 92 %). Although samples were stored and analysed separately, results for both post-dose days were combined for the purpose of studying the metabolic pattern of excretion.

Figure 4.3 depicts the pattern of metabolite excretion and reports the molar recovery of each metabolite per kg body weight for each dose. There was a significant increase in the total molar recovery after hydrolysis. The molar recoveries of Cy 5, 6 and 9 increased with hydrolysis after 0.37 mmol/kg *p*-cymene and Cy 2, 5, 8 and 9 increased after 1.49 mmol/kg *p*-cymene (Figure 4.3).

As for rats (Chapter 3), metabolite recoveries were standardised as the percentage of total recovered metabolites. The excretion of free, conjugated and total metabolites is reported in Table 4.1. Cy 5, a hydroxy acid, was the most abundant metabolite, accounting for about 60 % of total metabolites recovered after each dose. Cy 2, a structural isomer of Cy 5, accounted for only 4 and 8 % of the recovered metabolites after the low and high dose, respectively. Each of the other metabolites accounted for 6 - 20 % of the recovered metabolites, depending on the dose (Table 4.1).

There were some minor variations between the two doses in the pattern of total metabolites excreted. Cy 2 and 8 accounted for a greater percentage of the recovered metabolites after the higher dose, whereas Cy 6 accounted for a lower percentage after the higher dose.

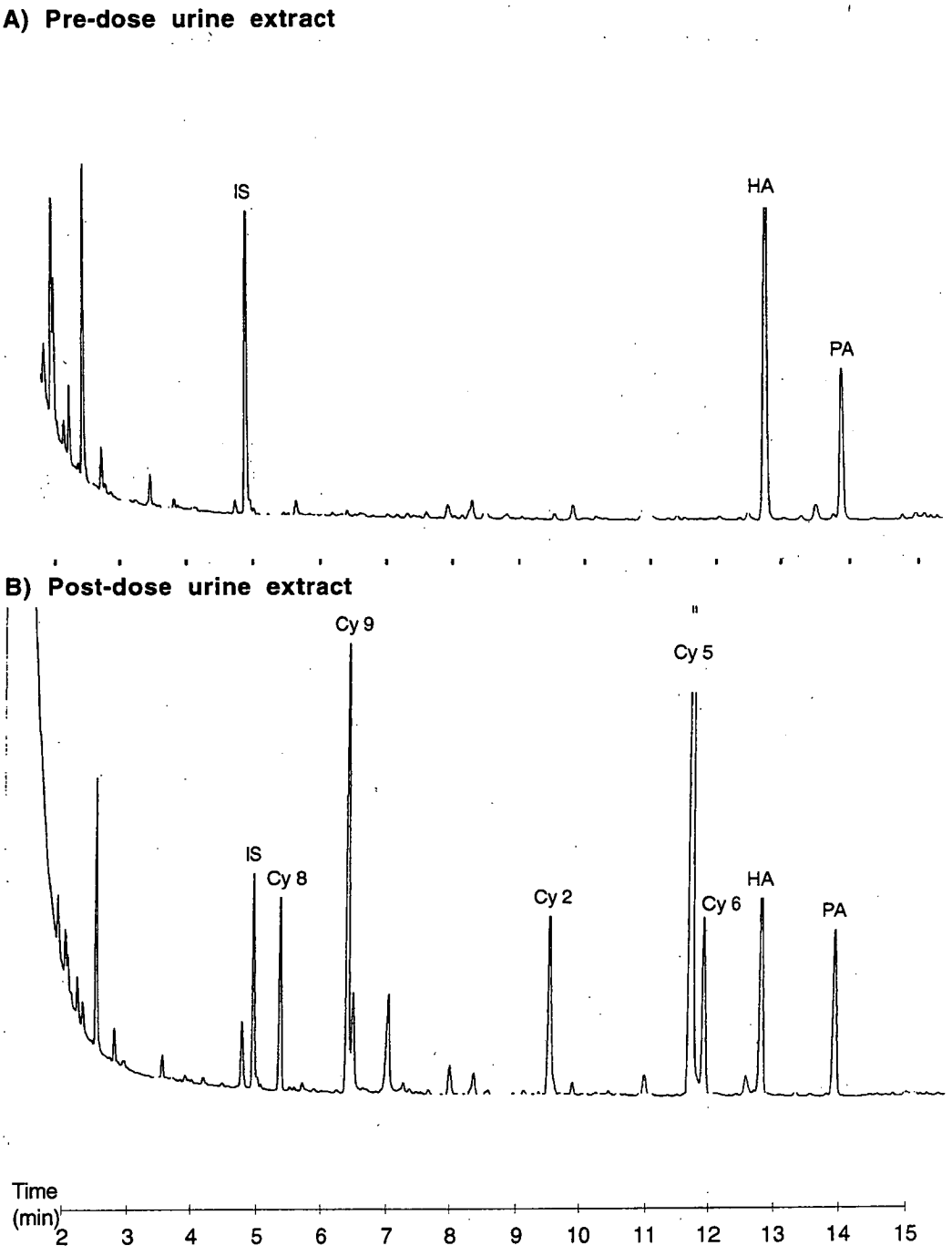
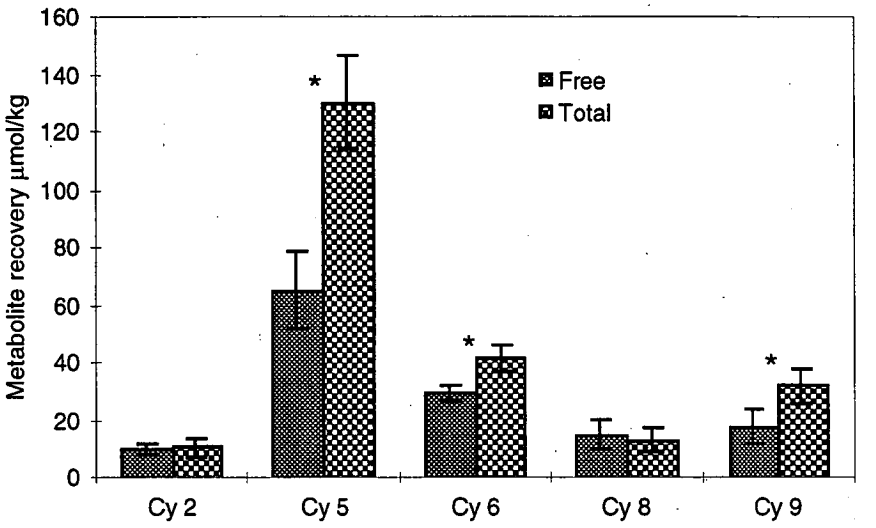


Figure 4.2. GC/FID chromatogram of pre- and post-dose hydrolysed urine extracts from the brushtail possum. Metabolites were derivatised to their methyl esters. Peaks labelled are IS = internal standard, HA = hippuric acid, PA = phenyl acetic acid and Cy 2, 5, 6, 8 and 9 are *p* -cymene metabolites. The urine extracts were from BP-1.

A) Dose 0.37 mmol/kg



B) Dose 1.49 mmol/kg

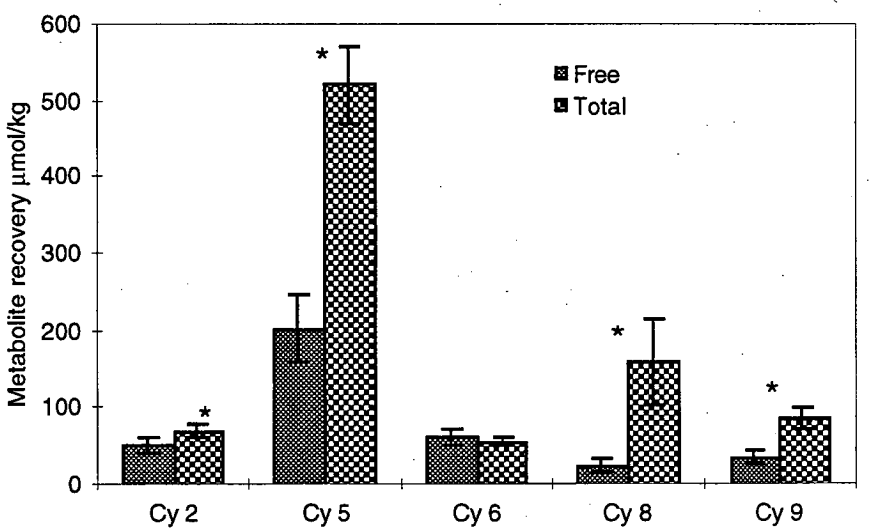


Figure 4.3. Molar recoveries (µmol/kg; mean ± se, n = 6) of free and total levels for each metabolites excreted by the brushtail possum after A) 0.37 mmol/kg *p* -cymene and B) 1.49 mmol/kg. Overall, total metabolite recovery was increased by hydrolysis (P = 0.005) and those individual metabolites whose recovery were significantly increased are indicated with an * (Student's paired t -test, $P < 0.05$).

There was no difference between the two doses in the overall percentage of the metabolites excreted as glucuronides, however, individual metabolites exhibited different degrees of conjugation. The majority of Cy 8 was conjugated after the higher *p*-cymene dose, while at the lower dose there was no conjugation. Cy 6 did not conjugate at all after the higher dose, whereas a small amount was conjugated after the lower dose.

Table 4.1. Excretion of free, conjugated and total metabolites expressed as a percentage of total recovered metabolites after each dose of *p* -cymene in the brushtail possum (n = 6).

	Percent (mean ± sd) of total urinary metabolites					
	Dose 0.37 mmol/kg			Dose 1.47 mmol/kg		
Metabolite	Free	Conjugated	Total	Free	Conjugated	Total
Cy 2	4.4 ± 1.8	1.0 ± 1.3	4.4 ± 2.3	6.1 ± 3.1	2.0 ± 1.5	8.0 ± 1.8
Cy 5	28.1 ± 9.0	28.7 ± 12.3	56.8 ± 5.4	23.8 ± 12.8	35.2 ± 16.8	59.0 ± 9.7
Cy 6	13.2 ± 3.4	5.2 ± 1.5	18.4 ± 3.8	7.7 ± 4.2	0.1 ± 0.1	6.6 ± 1.9
Cy 8	6.7 ± 6.5	2.2 ± 4.1	6.6 ± 5.8	2.8 ± 1.6	14.1 ± 9.4	16.9 ± 10.3
Cy 9	7.5 ± 4.3	6.3 ± 3.6	14.0 ± 2.9	4.0 ± 2.5	5.6 ± 3.2	10.0 ± 2.8
Sum	59.9† ± 18.2	40.1 ± 18.2‡	100†	44.3† ± 20.8	57.5 ± 20.8‡	100†

‡Comparison of percentage conjugated between doses, $P > 0.05$ (Student's paired t -test).
†Comparison of free and total recovery for each dose, $P < 0.05$ (Students paired t -test).
Each value is the mean ± sd of individual possums. Free and total metabolites were determined, respectively, before and after hydrolysis, and the difference was considered to be due to the conjugated metabolites. For some metabolites free levels were slightly greater than total, and the value of the difference was considered to be "0". Fractional recoveries of *p* -cymene were 0.62 ± 0.17 and 0.60 ± 0.14 for the low and high dose respectively.

Metabolites were grouped according to the number of oxygen atoms acquired during oxidation (Figure 4.4). Combined, Cy 2 and 5 (three oxygens) accounted for the largest portion (~ 65 %) of the recovered dose, with Cy 8 and 9 (two oxygens) together accounting for the next largest portion of the recovered dose. Cy 6 was the sole metabolite quantified with 4 oxygens.

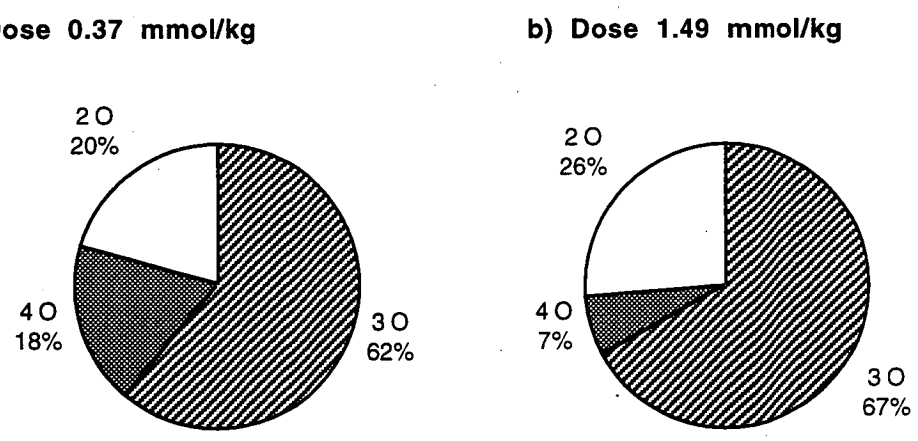


Figure 4.4. Diagramatic representation of metabolites grouped according to the number of oxygen (O) atoms acquired during oxidation and reported as the percent of recovered dose, after hydrolysis for A) 0.37 mmol/kg (n=6) and B) 1.49 mmol/kg (n=6) of *p* -cymene in brushtail possums.

4.3.3. Glucuronic acid excretion

There was a good correlation between post-dose molar glucuronic acid amounts and calculated molar metabolites conjugated ($R^2 = 0.80$, $P < 0.001$, $df = 22$). Attempts to reconcile conjugated metabolites with glucuronic acid measurements in individual possums were not successful as pre-dose values were unavailable and variability in the glucuronic acid assay was considerable. The urinary glucuronic acid concentrations are reported in Appendix 3 - Table A3.3.

4.3.4. Animal details

Individual possum details including weight, *p*-cymene dose, urine volumes and pH are recorded in Appendix 3 - Tables A3.2 and A3.4. The pH measurements of day 0 urine were alkaline, 8.0 ± 0.3 (mean ± sd), suggesting that the artificial diet fed to the possums resulted in a very low acid load. *p*-Cymene did not affect the pH measurements of post-dose urine samples, suggesting a considerable buffering capacity for organic acids. Fluid intakes and urine volumes were greater after the 1.49 mmol/kg dose than the 0.37 mmol/kg dose.

BP-5 and her pouch young were treated as a single animal. By the end of the experiment, the pouch young had emerged and was experimenting with eating but it still appeared to rely on its mother for the majority of its nourishment. The additional weight of the joey may have artificially exaggerated the mother's weight. However, the metabolism results of BP-5 showed no sign of being affected by the presence of the joey.

4.4. Discussion

The pattern of excretion of *p*-cymene metabolites was similar to that observed in the rat. A total of eight metabolites were identified of which five were present in sufficient amounts to quantify. The major metabolites excreted encompassed a range of oxidations (two to four oxygens being acquired), with moderately oxidised metabolites accounting for the majority of recovered metabolites. Conjugation of metabolites with glucuronic acid was significant, as approximately half of total metabolites had been conjugated.

The ultimate aim of xenobiotic metabolism is to produce the most readily excreted metabolites. Renal excretion is enhanced by increased polarity and whether this is achieved by extensive oxidation or conjugation will be dependent on metabolic adaptations. Metabolites of *p*-cymene are formed by serial oxidations occurring at one or more of three sites on the molecule. Cy 6, the dicarboxylic acid, is the ultimate of the oxidised metabolites, having acquired four oxygen atoms. Cy 4, 5, 8 and 9 are precursors of Cy 6, excreted before further oxidation could occur. Precursor, or intermediate, metabolites were excreted in both their free and conjugated forms. Oxidation occurred at both the C7 and C10 positions, although the dominance of Cy 5 in the metabolite profile would suggest that oxidation enzymes have a higher affinity for the C7 position. Cy 2 was oxidised at the C8 position also.

The fraction of the administered dose recovered was variable. A general discussion on the unaccounted portion of doses in each species is given in Chapter 8 - section 8.3.4. The percentage of the metabolites recovered in the first 24 h post-dose was much greater after the higher dose compared to the lower dose. Increased fluid intake after the higher dose may have accounted for the difference. The low recovery observed after the lower dose was exaggerated as one possum (BP-6) passed no urine in the first 24 h. Increased fluid intake, after the higher dose, resulted in more reliable and greater volumes of urine production. Combining metabolite recoveries from both days overcame variations such as this.

Trace amounts of cuminuric acid (Cy 10) and a second glycine conjugated metabolite, Cy 11, were detected in the urine. The glycine conjugation pathway was active, as the glycine conjugate of benzoic acid, hippuric acid, was excreted, yet this pathway was not involved to any significant extent in the detoxification of *p*-cymene in the brushtail possum.

The brushtail possum, being a generalist herbivore, naturally consumes a variety of food, including eucalypt leaves (Chapter 1). Generalist feeding behaviour is common among herbivores, as a varied diet results in ingestion of relatively small amounts of a large range of PSMs. Therefore a number of detoxification pathways, each with a low capacity, would be an advantage to the generalist herbivore. Freeland and Winter (1975) and Dearing and Cork (1999) have both demonstrated that brushtail possums are unable to maintain their body weight when fed a diet consisting solely of one species of eucalypt. They hypothesised that the cumulative dose of only a few PSMs exceeds the brushtail possum's detoxifying capacity which results in an inability to eat sufficient food to maintain body weight. Thus, it would seem the strategy employed by the brushtail possum requires a varied diet, in which the overall toxic load can potentially be great, but the detoxifying process is dispersed over numerous pathways. It is interesting that the results presented indicate a multiplicity of oxidative pathways in the metabolism of a single compound, *p*-cymene.

CHAPTER 5

P-CYMENE METABOLISM IN THE RINGTAIL POSSUM

5.1. Introduction

The metabolism of *p*-cymene in the ringtail possum was studied and is reported in this chapter.

Unfortunately, three out of six of the ringtail possums died in captivity. Full reports on each death are compiled in Appendix 4. There was no obvious cause of death in two possums. Because of the apparent vulnerability of the ringtail possums, data accumulated on the metabolism of *p*-cymene was limited to the three remaining possums.

5.2. Methods

5.2.1. Animals and Dosing

Six ringtail possums (weight 1.08 ± 0.18 kg (mean ± sd); 3 females and 3 males; identified as RT 1 - 6) were caught by hand from *Eucalyptus* bushland around the Hobart area. RT-5 had a pouch young which was included as part of its mother for the purpose of weighing and calculating *p*-cymene doses.

The possums were kept in large enclosures (2.5 m x 4 m x 2.5 m) at the University of Tasmania's Animal House. A maximum of two possums were kept in each enclosure and an excess of nest boxes were provided. The enclosures were furnished with aerial branches to allow animals to move around. Initially, while possums were adjusting to captivity, they were fed fresh leaf from *E. pulchella*, collected from the University grounds daily. The branch stems were immersed in water. Fresh water was also provided in trays on the floor of the enclosure. Two possums died during the acclimatisation period.

Possums were introduced to an artificial diet over several weeks. The diet was prepared fresh each day and was based on a diet used successfully by researchers at James Cook University (Table 5.1). All ingredients were mixed together in a bucket by hand and weighed amounts offered to possums each night.

The possums preferred *E. pulchella* leaves to the artificial diet and were reluctant to transfer to the artificial diet. The transition to artificial diet took up to 2 months for some possums.

Table 5.1. Artificial diet fed to ringtail possums.

Ingredients (preparation)	Weight g
Apples (grated)	700
Bananas (mashed)	360
Weetbix[1]	95
Lucerne chaff (ground to pass through a 2 mm sieve)	60
Rice hulls (ground to pass through a 2 mm sieve)	35
Casein - soluble	20
Honey	50

[1]Wheat based breakfast cereal (Sanitarium Health Food Company, Berkeley Vale, NSW)

The possums were transferred to metabolism cages prior to being dosed. The cages were standard rabbit metabolism cages, constructed from stainless steel (60 x 60 x 40 cm). Stainless steel splash guards were inserted on all sides to minimise losses of urine. Wooden nest boxes with mesh floors were suspended from the ceiling of each cage. Faeces were separated from the urine by a stainless steel grating placed over the collecting funnel. Urine was collected into measuring cylinders sitting in ice-filled thermos flasks. Urine volume and pH were measured and then the urine was frozen at -18°C until analysed. After each collection period, the floor and funnel of the cages were washed with distilled water and the washings added to the urine volume. Faeces were removed from the meshing and stored in specimen jars and frozen until analysed. A 24 h pre-dose urine and faeces sample was collected and then two 24 h post-dose collections made.

Initially, three apparently healthy possums were administered *p*-cymene (1.49 mmol/kg). The lower dose was originally omitted to minimise time and stress on possums. Experience gained from dosing other species suggested that the possums would readily tolerate the larger dose. Unfortunately, the third death (RT-3) occurred within 24 h of the dose and urine from another possum (RT-2) was lost, leaving usable results from only one possum (RT-5). The remaining possums were successfully administered the lower dose of *p*-cymene (0.37 mmol/kg). A *p*-cymene mixture in peanut oil was prepared (0.22 mmol/ml) to provide a dose volume of less than 2 ml. Doses were administered by oral gavage using a flexible paediatric feeding tube. The dosing technique and the environemtal conditions were the same as described in Chapter 4 - section 4.2.1.

The remaining ringtail possums were released at their point of capture at the end of the experiments.

5.2.2. Urine analyses

A 0.5 ml urine sample from RT-5 after the 1.49 mmol/kg dose of *p*-cymene was diluted (1:2) and 1 ml of undiluted urine from all three possums (RT-2, 4 and 5) after the 0.37 mmol/kg dosing were analysed for both free and total metabolites and the major metabolites quantified as described in Chapter 2 - section 2.2.1.

5.2.3. Identification of metabolites

Metabolites were identified by their GC retention times and confirmed by their respective mass spectra using GC/MS.

5.2.4. Quantitation of urinary metabolites

Cy 2, 3 and 6 were present in sufficient amounts to quantify (see Figure 5.1. and Chapter 2 -Table 2.1 for chemical structures and names of metabolites). Cy 5 was present as a trace amount only. Each of these metabolites was isolated from ringtail possum urine, collected during the dosing experiments.

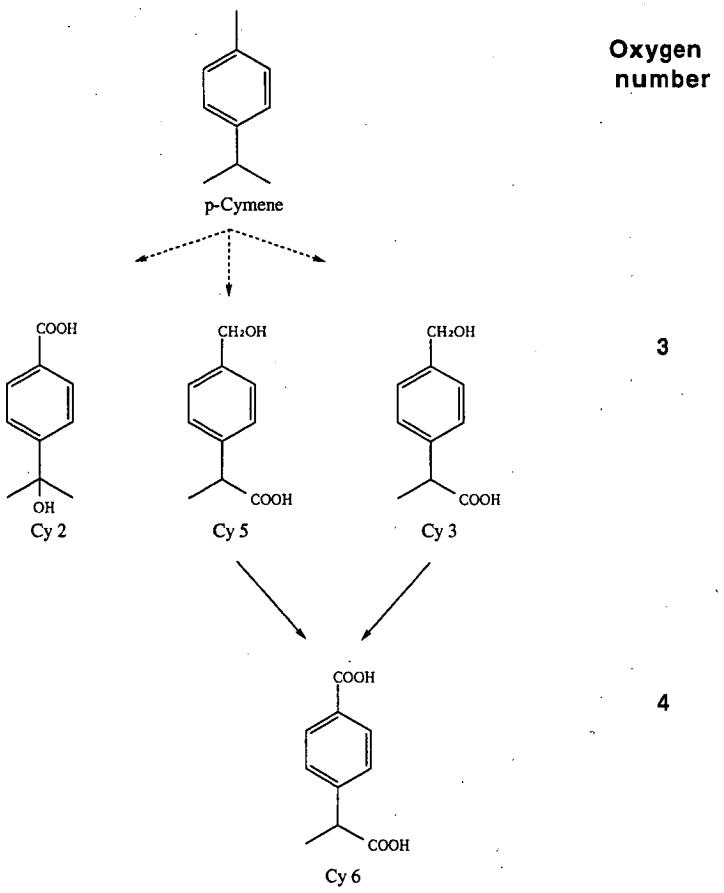


Figure 5.1. Chemical structures and proposed metabolic pathways of *p* -cymene metabolites in the ringtail possum. Metabolites are shown in their underivatised forms. Dashed arrows indicate where precursor metabolites have not been detected and number of oxygen atoms acquired are shown on the right side of the diagram. See Chapter 2 - Table 2.1 for chemical names. Cy 5 was found as a trace only in the greater glider.

Preliminary pTLC was used to separate metabolites, followed by a secondary pTLC of each metabolite, producing the final standards for quantitation (methods described in Chapter 2 - section 2.2.4). The purities for isolated Cy 2, 3 and 6, by GC/FID, were 100, 85 and 97.5 % respectively. Impurities in metabolite isolates were derived from other minor urinary components, none of which interfered with metabolite peaks. Calibration curve dilutions of metabolites were adjusted for weight to account for impurities.

Calibration curves were prepared for Cy 2, 3 and 6 as described in Chapter 2 - section 2.2.5.1.4 and calibration curve parameters are reported in Appendix 5 - Table A5.1 and were linear over the required concentration ranges.

5.2.5. Urinary glucuronic acid assay

Full 24 h urine samples were collected for each ringtail possum allowing direct comparison of urinary glucuronic acid output between pre- and post-dose samples. Analysis of urinary glucuronic acid was described in Chapter 2 - section 2.2.6.

5.3. Results

5.3.1. Qualitative results

Figure 5.2 shows a typical gas chromatogram of pre- and post-dose urine extracts. *p*-Cymene metabolites were the major extractable components of ringtail possum urine.

Four metabolites, Cy 2, 3, 5 and 6 were identified in the urine of the ringtail possum (see Figure 5.2 for chemical structures and names). Cy 2, 5 and 6 were familiar metabolites, having been identified as significant metabolites in all species reported in this study. Cy 3, however, was only detected in the rat urine as a trace metabolite and was identified from the mass spectra published by Walde *et al.* (1983). Mass spectra for each of these metabolites are reported in Chapter 2 - Table 2.6.

5.3.2. Quantitative results

p-Cymene metabolites were quantified in urine from three possums (RT-2, 4 and 5) after 0.37 mmol/kg of *p*-cymene and in one possum (RT-5) after the 1.49 mmol/kg dose. Fractional recoveries of the administered doses were 0.74 ± 0.07 (mean ± sd; range 0.65 - 0.79) and 0.87 for the low and high doses, respectively. The percentage of the lower dose recovered in the first 24 h was variable between the three possums, reflecting variation in urinary output. RT-2, 4 and 5 excreted 81, 45 and 97 % of the dose in the first 24 h, respectively. After 1.49 mmol/kg, RT-5 excreted 93 % of the recovered metabolites in the first 24 h. Samples were stored and analysed separately and the results for both post-dose days were combined.

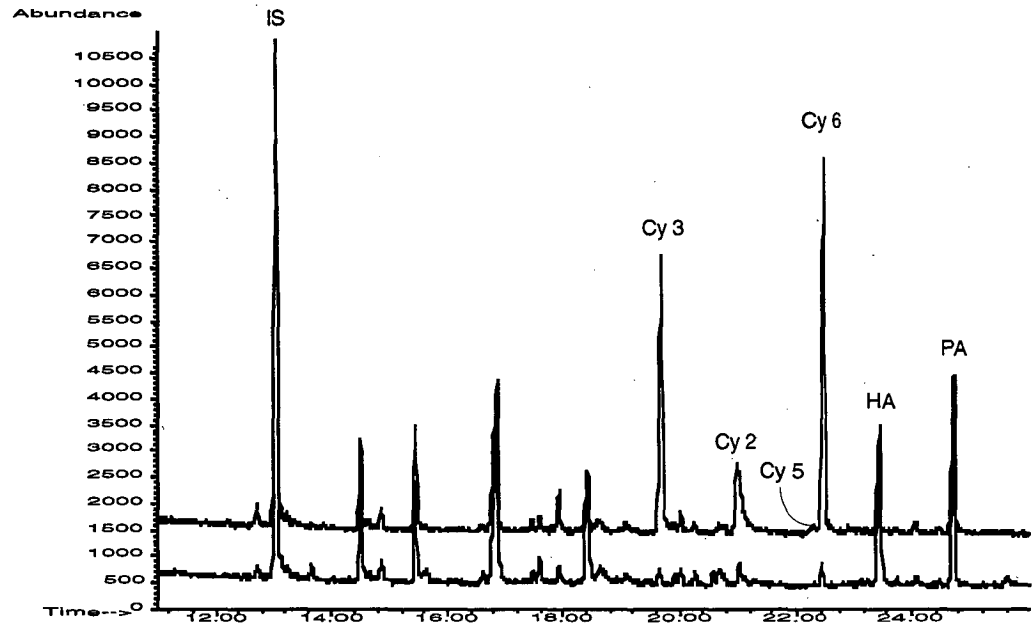


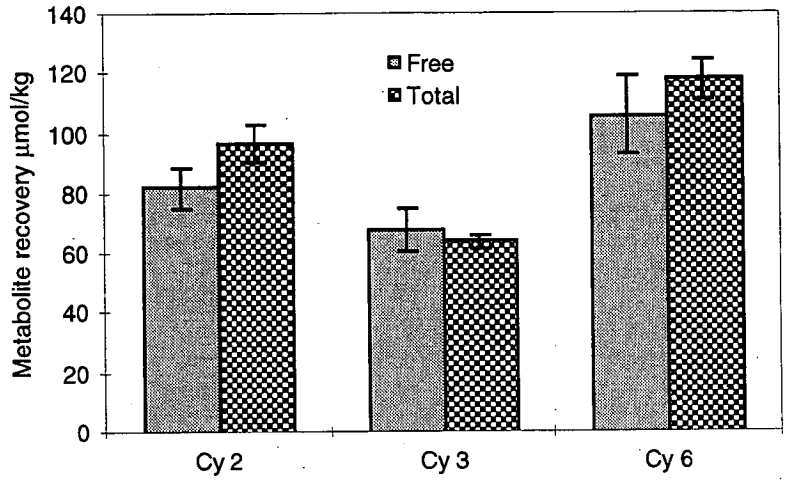
Figure 5.2. GC/FID chromatogram of pre- (bottom) and post- (top) dose hydrolysed urine extracts from the ringtail possum. Metabolites are derivatised to their methyl esters. Peaks labelled are IS = internal standard, HA = hippuric acid, PA = phenyl acetic acid and Cy 2, 3, 5 and 6 are *p* -cymene metabolites.

Figure 5.3 depicts the pattern of metabolite excretion and reports the molar recovery of each metabolite per body weight (kg^{-1}). There was no significant increase in the recovery of any metabolite after hydrolysis, suggesting there was no significant hydrolysable conjugation of *p*-cymene metabolites.

Metabolite recoveries were expressed as the percentage of total recovered metabolites in Table 5.2. Cy 6, the dicarboxylic acid, was the most abundant metabolite, accounting for 42 % of the total metabolite. Cy 2, which acquires one less oxygen than Cy 6, was the next most significant metabolite (35 %) and Cy 3, an isomer of Cy 2, was the least abundant metabolite (23 %).

Although the excretion pattern of metabolites after 1.49 mmol/kg is reported for only one possum, there seems to be little variation in the pattern of metabolite excretion dependent on dose.

A) Dose 0.37 mmol/kg



B) Dose 1.49 mmol/kg

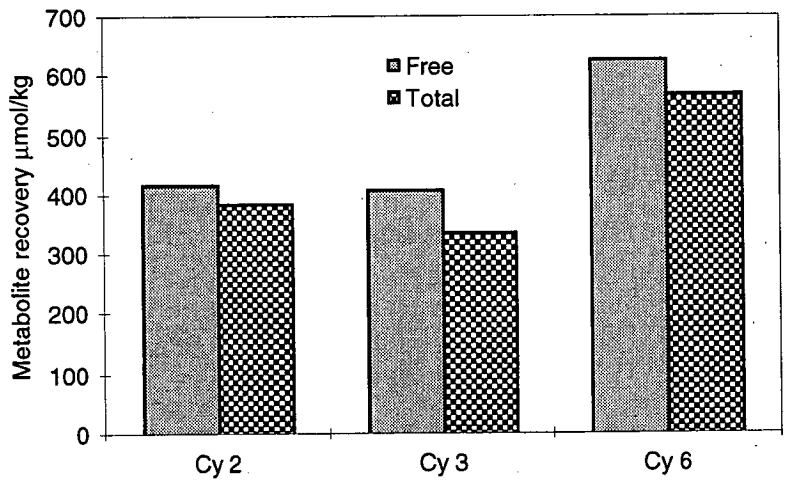


Figure 5.3. Molar recoveries (µmol/kg; mean ± se) for free and total levels of each metabolite excreted by the ringtail possum after each dose. A) 0.37 mmol/kg (n=3) and B) 1.49 mmol/kg (n=1). No significant conjugation of *p* -cymene metabolites was detected (Student's paired *t* -test, $P > 0.05$).

Table 5.2. Excretion of free, conjugated and total metabolites expressed as a percentage of total recovered metabolites after *p* -cymene in the ringtail possum.

	Percent (mean ± sd) of total urinary metabolites					
	Dose 0.37 mmol/kg (n = 3)			Dose 1.47 mmol/kg (n = 1)		
Metabolite	Free	Conjugated	Total	Free	Conjugated	Total
Cy 2	29.4 ± 2.0	5.3 ± 4.1	34.7 ± 4.7	32.3	0	29.9
Cy 3	24.5 ± 4.5	0 ± 0.3	23.2 ± 2.4	32.1	0	26.1
Cy 6	37.9 ± 6.6	4.3 ± 3.3	42.1 ± 3.6	48.6	0	44
Sum	91.7 ± 9.5	9.1 ± 8.0	100 ± 0.0	113	0	100

Comparison of percentage free and total after 0.37 mmol/kg, $P > 0.05$ (Student's paired t -test).
Each value is the mean ± sd of individual possums. Free and total metabolites were determined, respectively, before and after hydrolysis, and the difference was considered to be due to the conjugated metabolites. For some metabolites free levels were slightly greater than total, and the value of the difference was considered to be "0". Fractional recoveries of *p* -cymene were 0.74 ± 0.07 and 0.87 (n = 1) for the low and high dose respectively.

Metabolites were grouped according to the number of oxygen atoms acquired during oxidation (Figure 5.4). Combined, Cy 2 and 3 (three oxygens) accounted for the largest portion (68 %) of the recovered dose, with Cy 6 (four oxygens) accounting for the remainder.

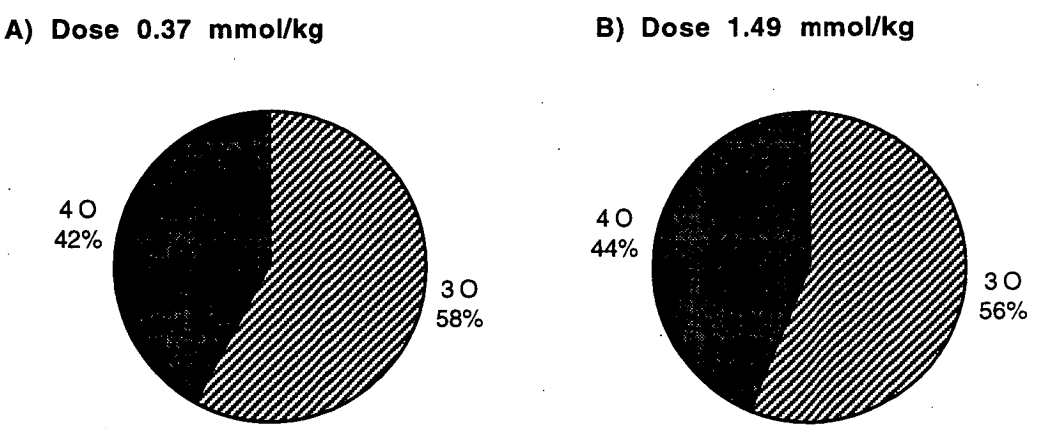


Figure 5.4. Metabolites grouped according to the number of oxygen (O) atoms acquired during oxidation and reported as the percent of recovered dose, after hydrolysis.

5.3.3. Glucuronic acid excretion

Glucuronic acid levels were low in all urine samples (Appendix 5 - Table A5.3). Glucuronic acid measurements support the observation that conjugation of *p*-cymene metabolites was insignificant in the ringtail possum. Furthermore, few urinary glucuronides were excreted by ringtail possums fed the artificial diet. There was, however, a reasonable correlation between food intake (g/kg) and glucuronic acid (µmol/kg) levels ($R^2 = 0.65$, $P < 0.05$, $df = 8$).

5.3.4. Animal details

Individual possum details including weight, *p*-cymene dose, recovery of each metabolite and urine volumes and pH are recorded in Appendix 5 - Tables A5.2 and A5.4. The pH measurements of fresh urine were very alkaline, 8.0 ± 0.9 (mean ± sd), suggesting that the artificial diet fed to the possums resulted in a very low acid load. The *p*-cymene dose did not cause a measurable reduction in pH (Anova (single factor) $df = 2$, $F = 0.47$ and $P = 0.64$).

Intake of artifical diet for each possum on experimental days is reported in Appendix 5 - Table A5.5.

5.4. Discussion

The pattern of metabolite excretion in ringtail possums was markedly different to that observed in rat and brushtail possum. The ringtail possum excreted only three metabolites (with traces of a fourth, Cy 5), compared to nine and eight respectively in the rat and brushtail possum. Each metabolite had acquired a minimum of three oxygens during oxidation, resulting in extensively oxidised and therefore polar metabolites. Subsequent conjugation with glucuronic acid was not required for renal elimination of the polar metabolic products.

Both urinary glucuronic acid measurements and the difference in total and free metabolite recovery supported this observation. Urinary glucuronic acid measurements were uniformly low, indicating that no component of the artificial diet required conjugation. McLean *et al.* (1993) also reported that ringtail possums fed an artificial diet excreted low levels of glucuronic acid, but this increased when their diet was changed to *E. radiata* leaf.

The urine collected from ringtail possums was extremely alkaline. Urine pHs were above 8. The acidic *p*-cymene metabolites had little impact on the urine pH. It would seem that ringtail possum urine has a large buffering capacity which would be important in acid-base homeostasis when feeding on a leaf diet where acidic metabolites are eliminated. Unfortunately urine samples were not collected from ringtails feeding on *E. pulchella*, therefore the effect of the *E. pulchella* on urine pH was not determined directly.

The fraction of the administered dose recovered is acceptable, however, a general discussion on the unaccounted portions of doses in all species is given in Chapter 8 - section 8.3.4.

The health and condition of possums was very concerning throughout this study. No link could be established between the three deaths, and apart from RT-6, which was treated for a cloacal abscess, no firm cause of death was established. The death of the first two possums occurred during their acclimatisation to captivity and artificial diet. Therefore it would seem that the health of these possums, at the time of capture, may have been poor. The autopsy on RT-3 concluded that the dose of *p*-cymene itself was not responsible for the death. A fourth possum, RT-4, also had periods of bad health, developing sores around the base of the tail as a result of

gnawing at the skin. Details and autopsy reports for each of the deaths are included in Appendix 4.

CHAPTER 6

P-CYMENE METABOLISM IN THE GREATER GLIDER

6.1. Introduction

The metabolism of *p*-cymene in the greater glider (*Petauroides volans*) was studied as part of the overall comparison of its metabolism in marsupial eucalypt folivores.

6.2. Methods

6.2.1. Animals and Dosing

Six greater gliders (weight 0.66 ± 0.70 kg (mean ± sd); 5 females and 1 male; identified as GG A-F) were caught by hand in *Eucalyptus* forest near Townsville, Queensland.

Gliders were housed at the Department of Zoology and Tropical Ecology, James Cook University, Townsville and released back to their point of capture at the end of the experiments. Gliders were held in individual wire mesh enclosures (dimensions 1.5 m x 0.6 m x 0.9 m) during their captivity and transferred to metabolism cages for dosing experiments. Each enclosure was furnished with aerial branches and a nest box.

The greater glider is an obligate eucalypt folivore, therefore it was not possible to transfer animals to an artificial diet. Between dosing experiments gliders were offered a mixed diet from the following species: *E. crebra, E. tenuranis, E. (=Corymbia) citriodora, E. (=Corymbia) polycarpa* and *E. tereticornis*. The day before dosing, gliders were offered solely *E. polycarpa* foliage as this leaf was found, by analysis, to contain < 0.1 % total terpenes and no *p*-cymene.

Fresh leaves were collected every few days from eucalypt forests in the Townsville region and stored in a cold room until required. Gliders were supplied with fresh leaf and water containing 7 % dextrose daily. Food and water intakes were monitored daily and gliders were weighed at regular intervals.

Gliders are vulnerable to stress when in captivity (Dr W. J. Foley, personal observations). For this reason several modifications of experimental procedure were made. First, only one dose of *p*-cymene was administered to the gliders, since the metabolism of *p*-cymene in the other species indicated minimal dose dependency.

Second, a full 24 h pre-dose urine sample was replaced with a small sample of urine collected immediately prior to dosing. This reduced the residence time in the metabolism cage to 48 h. A sample of control urine was considered satisfactory since its main purpose was to confirm the absence of *p*-cymene metabolites. Comparisons in pre- and post-dose glucuronic acid concentrations were made by normalising

glucuronic acid with creatinine concentrations. Control urine and faeces samples were obtained by stroking the cloaca which stimulated both defecation and urination. Urine was funnelled directly into sample bottles.

Gliders were administered a single dose of *p*-cymene (1.49 mmol/kg) in peanut oil. A *p*-cymene mixture was prepared (1.12 mmol/ml) to provide a dose volume of approximately one ml. The dose was administered by oral gavage using a flexible paediatric feeding tube, as described in the dosing of brushtail possums in Chapter 4 - section 4.2.1.

Gliders were transferred to metabolism cages immediately after dosing. The cages were custom made from plastic coated wire meshing. Stainless steel splash guards were inserted on all sides. Nest boxes, with mesh floors, were also installed into each cage. Branches of *E. polycarpa* were pruned until only the preferred young edible leaf remained. The branches were carefully arranged in the cages in such a way to minimise losses of urine and faeces onto leaf.

Two 24 h post-dose collections of urine and faeces were made (days 1 and 2). Faeces were separated from the urine by nylon mesh netting across the collecting funnel. Urine was frozen immediately by collecting into plastic screw cap bottles (100 ml) inserted into a thermos flask filled with solid CO_2. The urine was kept frozen at -18°C until analysed. Faeces were removed from the meshing and stored in specimen jars and frozen until analysed.

After each collection period, residual urine was washed from the collecting funnels with distilled water and the washings added to the frozen urine.

Urine samples were thawed in the laboratory prior to analysis and total volume and pH measured. Faeces were manually separated from leaf scraps and weighed before freezing.

Animals were kept in a temperature and light controlled room throughout their captivity. The room was thermostatically controlled at 19°C (minimum and maximum temperatures were recorded daily and ranged from 16 - 23°C over a two week period). A 12 h light/dark cycle was used with gradation of light simulating dawn and dusk. The floor and walls of the room were hosed with water twice daily to maximise humidity in the room to assist in maintaining leaf quality.

6.2.2. *E. polycarpa* leaf analysis

The terpene content and profile of *E. polycarpa* was investigated by ethanolic extraction of leaf samples. Six samples from different batches of collected leaf were analysed. To extract terpenes, a weighed amount of fresh leaf (approximately 5 g) was placed into a 50 ml plastic centrifuge tube with 50 ml of ethanol (99%) and stood at room temperature for 48 h before analysis (Ammon *et al.* 1985).

GC/MS was used to analyze the leaf extracts. The instrument and capillary column used was described in Chapter 2 - section 2.2.2. GC/MS operating conditions were: splitless injector 250°C, He carrier gas (15 psi), oven 60 - 190°C at 5°C/min, 190 - 290°C at 30°C/min and held for 5 min, detector 290°C. The TIC chromatogram of a typical leaf extract is shown in Figure 6.1. The monoterpenes identified in the leaves

were α-pinene, β-pinene and 1,8-cineole. Several other minor peaks were not identified but were also likely to be terpenoid. Other compounds dominated the extractable content of the leaf, and were likely to be sesquiterpenes.

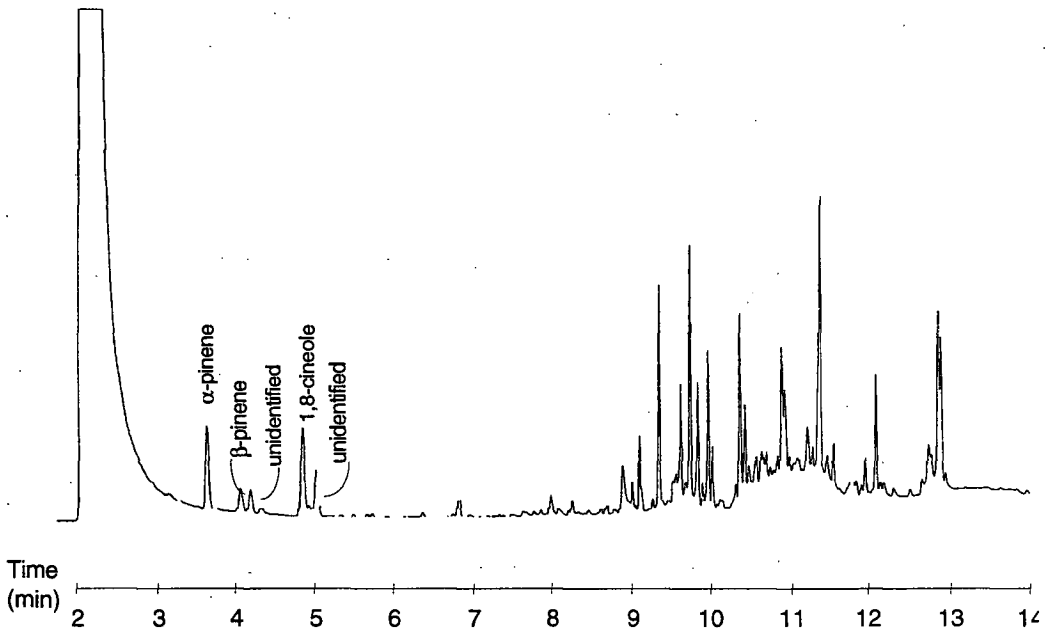


Figure 6.1. GC chromatogram of ethanolic extract of *E. polycarpa* leaves fed to greater gliders. Terpene content was low (ranging from 0.03 - 0.06 % wet weight) however other larger extractable compounds were present.

The terpene content of *E. polycarpa* was estimated from a known amount of an internal standard (*p*-cymene) and was based on the assumption that each terpene would have a similar GC/MS response factor (Jorgensen *et al.* 1990). An ethanolic stock solution of *p*-cymene (5 mg/ml) was prepared and 1 ml of this added to 5 ml of each leaf extract. The sample was concentrated by evaporation to produce a final volume of approximately 1 ml. This was analysed by GC/MS. The total leaf monoterpene content was quantified relative to the internal standard. The estimated monoterpene concentrations were very low, ranging from 0.025 - 0.058 % wet weight of the leaf.

6.2.3. Urine analyses

Free and total urinary metabolites were analysed in greater glider urine collected after 1.49 mmol/kg of *p*-cymene. One ml of diluted urine (1:2) was used for the analyses and the method is described in Chapter 2 - section 2.2.1.

Metabolites were identified by their GC retention times and confirmed by their respective mass spectra using GC/MS.

6.2.4. Quantitation of urinary metabolites

The metabolites to be quantified were Cy 2, 5 and 6 (Figure 6.2. and Chapter 2 -Table 2.1 for chemical structures and names of metabolites). Because of the contaminating influence of other urinary components in the glider urine, the required metabolites were isolated from urine from brushtail possums. Two stage pTLC was used to isolate and purify the metabolites as described in Chapter 2 - section 2.2.4.

Calibration curves were prepared for Cy 2, 5 and 6 as described in Chapter 2 - section 2.2.5.1. The purities for isolated Cy 2, 5 and 6 were 78, 95 and 91 % respectively. Impurities in each metabolite isolated did not interfere with metabolite peaks. Calibration curve parameters are reported in Appendix 6 - Table A6.1 and were linear over the required concentration ranges.

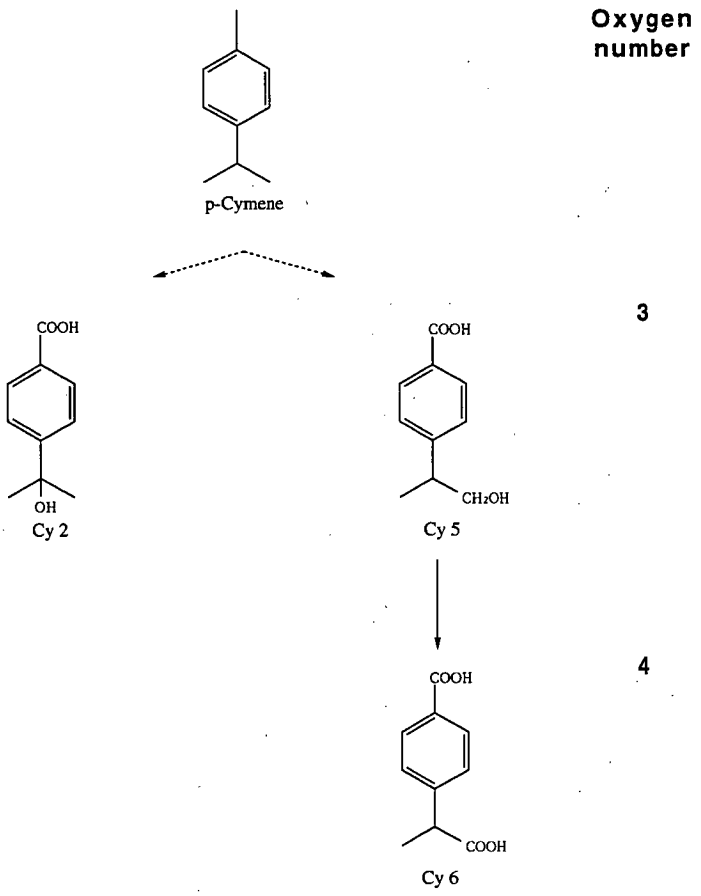


Figure 6.2. Chemical structures and proposed metabolic pathway of *p* -cymene metabolites in the greater glider. Metabolites are shown in their underivatised forms. Dashed arrows indicate where precursor metabolites have not been detected.

6.2.5. Urinary glucuronic acid analysis

Pre-dose urine samples were not full 24 h collections. Therefore direct comparisons between pre- and post-dose urinary glucuronic acid concentrations could not be made. Instead, glucuronic acid concentrations were standardised with urinary creatinine concentrations to provide a comparison between the pre- and post-dose urine. The method used to measure urinary creatinine was based upon Jaffe's reaction with picric acid (Clark and Thompson 1949; Jacobson 1996).

Urinary creatinine assay. Initially a calibration curve for creatinine concentrations was prepared by making a stock solution of creatinine (17 µmol/ml in 0.1 M HCl) which was further diluted by a 1:50 dilution with water. Aliquots of 10, 25, 50, 100, 250, 500 and 1000 µl were measured into clean centrifuge tubes and made to a final volume of 2.01 ml with water. To each tube was added 1 ml of picric acid (13.6 g/l) and 0.5 ml of 1.4 M NaOH solution and the contents mixed thoroughly on a vortex mixer. The absorbance at 500 nm was measured for each dilution against a blank (2.01 ml water + 1 ml picric acid solution + 0.5 ml 1.4 M NaOH) exactly 15 min after the addition of the picric acid and NaOH using a Cecil Instruments CE20 ultraviolet spectrophotometer. The creatinine calibration curve was linear over the concentration ranges measured (1.78 to 178 µmol/l).

Creatinine concentrations in glider urine were assayed in the same manner, except creatinine dilutions were replaced with diluted urine (1:50).

Urinary glucuronic acid: Glucuronic acid was measured in all urine samples as described in Chapter 2 - section 2.2.6. Greater glider urine was diluted a thousand-fold for the analysis. Glucuronic acid concentrations were divided by the corresponding creatinine concentration resulting in a ratio allowing a comparison of glucuronic acid output in pre- and post-dose urine samples.

6.3. Results

6.3.1. Qualitative results

Figure 6.3 (A and B) shows the GC/FID chromatogram of hydrolysed urine samples pre- and post-dose. Although overwhelmed by other urinary compounds, *p*-cymene metabolites eluted separately, with no interference from the major peaks. The major urinary compounds were isomers of hydroxy and methyl benzoic acids, metabolites of other leaf compounds present in the diet.

Three metabolites, Cy 2, 5 and 6, were identified in the urine of greater gliders (Figure 6.2). All metabolites have been identified in other animal species and reported in the literature (Southwell *et al.* 1980; Ishida *et al.* 1981; Walde *et al.* 1983). The probable metabolic pathway is shown in Figure 6.2.

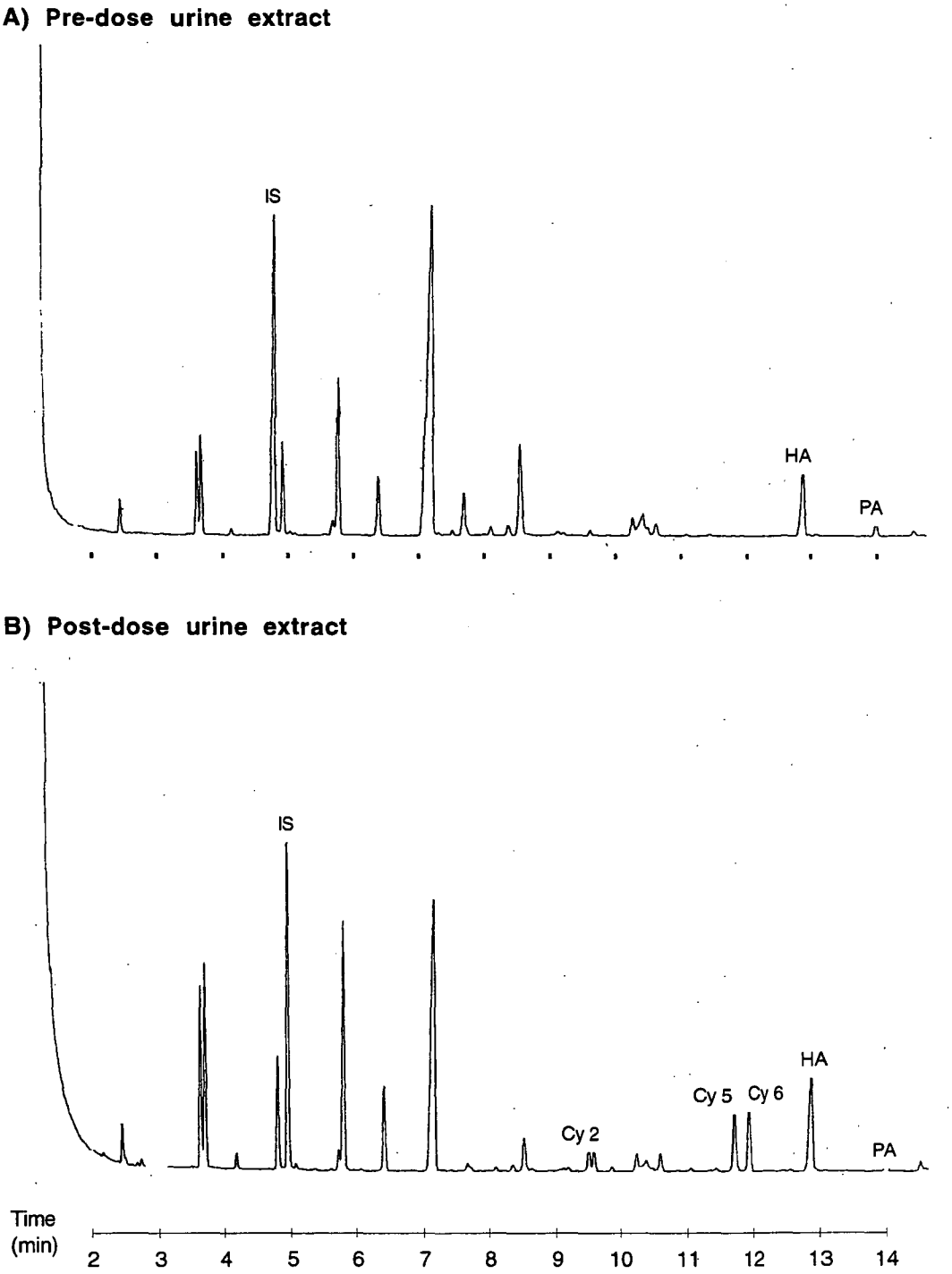


Figure 6.3. GC/FID chromatogram of pre- and post-dose hydrolysed urine extracts from the greater glider. Metabolites are derivatised to their methyl esters. Peaks labelled are IS = internal standard, HA = hippuric acid, PA = phenyl acetic acid and Cy 2, 5 and 6 are *p* - cymene metabolites. Urine samples were from GG - E.

6.3.2. Quantitative results

All three metabolites were present in quantities sufficient to measure. The total fraction of the administered dose of 1.49 mmol/kg *p*-cymene recovered in 48 h was 0.74 ± 0.07 (mean ± sd; n = 6; range 0.62 - 0.84). Most of the metabolites were recovered in the first 24 h post-dose (92 ± 8 % (mean ± sd); range 84 - 100 %). Samples were stored and analysed separately. As in the analysis of urine from the other species, total recoveries were considered by combining the results for each day.

Figure 6.4 depicts the pattern of metabolite excretion and reports the molar recovery of each metabolite per body weight (kg^{-1}). There was no significant increase in the recovery of any metabolite after hydrolysis (Student's paired *t*-test, $P > 0.05$ in each case), indicating there was no significant hydrolysable conjugation of *p*-cymene metabolites.

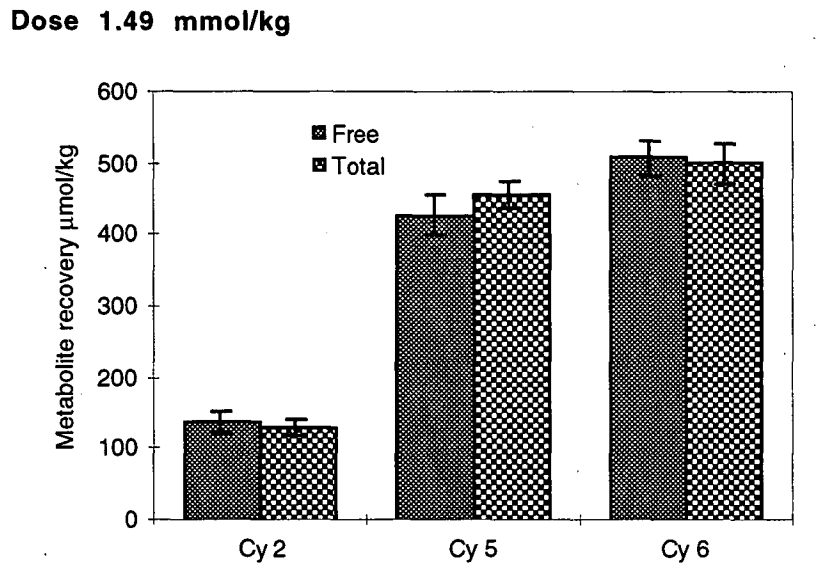


Figure 6.4. Molar recoveries (mmol/kg; mean ± se, n = 6) of free and total levels for each metabolite excreted by the greater glider. No significant conjugation of *p* -cymene metabolites was detected (Student's paired *t* -test, $P > 0.05$).

The excretion of free, conjugated and total metabolites are expressed as the percentage of total recovered metabolites and are reported in Table 6.1. The extensively oxidised dicarboxylic acid, Cy 6, was the most abundant metabolite, accounting for almost half of the recovered dose. Cy 5, which acquires 3 oxygen atoms during oxidation, also accounted for a large portion (about 40 %) of the recovered dose. Cy 2, a structural isomer of Cy 5, was the only other metabolite found.

Table 6.1. Excretion of free, conjugated and total metabolites expressed as a percentage of total recovered metabolites after *p* -cymene in the greater glider (n = 6).

	Percent (mean ± sd) of total urinary metabolites		
	Dose 0.37 mmol/kg		
Metabolite	Free	Conjugated	Total
Cy 2	12.4 ± 2.8	0.7 ± 1.2	12.8 ± 2.2
Cy 5	43.0 ± 5.6	4.7 ± 4.9	39.7 ± 3.2
Cy 6	47.0 ± 7.0	1.5 ± 2.4	47.5 ± 4.4
Sum	102.4 ± 13.2	5.9 ± 9.0	100 ± 0.0

Comparison of percentage free and total after 0.37 mmol/kg, $P > 0.05$ (Student's paired t -test).
Each value is the mean ± sd of individual gliders. Free and total metabolites were determined in separate analyses, before and after hydrolysis, respectively. The difference was considered to be due to the conjugated metabolites.
For some metabolites free levels were slightly greater than total, and the value of the difference was considered to be "0", and
Fractional recovery of *p* -cymene was 0.74 ± 0.07.

Metabolites were grouped according to the number of oxygen atoms acquired during oxidation (Figure 6.5). Combined, Cy 2 and 5 (three oxygens) accounted for the largest portion (53 %) of the recovered dose, with Cy 6 (four oxygens) accounting for the remaining 47 % of the recovered dose.

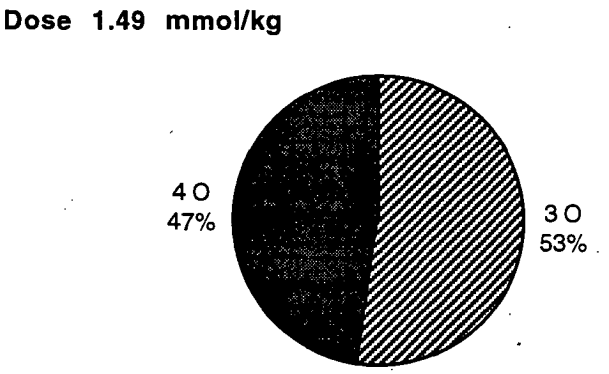


Figure 6.5. Metabolites grouped according to the number of oxygen (O) atoms acquired during oxidation and reported as the percent of recovered dose after hydrolysis.

6.3.3. Glucuronic acid excretion

There was no significant difference in the glucuronic acid to creatinine ratio between pre- and post-dose measurements (Appendix 6 - Table A6.3). Significant amounts of glucuronic acid were excreted before and after the *p*-cymene dose. Therefore glucuronic acid measurements support the observation that conjugation of *p*-cymene metabolites was insignificant in the greater glider.

6.3.4. Animal details

Individual possum details including *p*-cymene dose, molar recovery of each metabolite and urine volumes and pH and weights are recorded in Appendix 6 - Tables A6.2, A6.4 and A6.5. Pre- and post dose urine volumes could not be compared as a full 24 h pre-dose samples were not collected. Neat urine volumes were not measured in the post-dose collections, as the urine was frozen immediately and the cage washings were added to the frozen urine. pH measurements on fresh pre-dose urine samples were relatively acidic, 5.4 ± 0.2 (mean ± sd).

Leaf intake was 134 ± 33 g/kg/day (mean ± sd) for gliders feeding on the mixed eucalypt leaf diet. While in the metabolism cages feeding on *E. polycarpa* alone they ate significantly less leaf (103 ± 20 g/kg/day (mean ± sd); Anova (single factor) $df = 1, F = 13, P < 0.001$).

GG B became ill after dosing. She consumed no food or water in the 24 h after dosing and was removed from the metabolism cage to be nursed back to her original weight.

6.4. Discussion

The metabolic strategy employed by the greater glider to detoxify *p*-cymene promotes the rapid production and elimination of extensively oxidised metabolites. The pattern of excretion of *p*-cymene metabolites in the gliders was relatively simple with only three metabolites being excreted, all of which were extensively oxidised, acquiring at least three oxygen atoms. No significant glucuronidation was detected by hydrolysis. The molar amounts of glucuronic acid generally exceeded the overall *p*-cymene dose. There was no significant difference in the urinary glucuronic acid/creatinine ratio between pre- and post dose. Large variations in measured values suggest that these determinations would be insensitive to minor amounts of *p*-cymene conjugation. It would appear the excretion of glucuronic acid is unassociated with the excretion of *p*-cymene in greater gliders.

Evidently the glider has a similar pattern of metabolism to the ringtail possum. Both excrete only extensively oxidised metabolites which do not require conjugation to be eliminated. There was, however, an important difference in experimental conditions between the two species. Ringtails were maintained on an artificial diet whereas the gliders were fed a leaf diet. This may result in the induction of oxidation enzymes in the glider while the enzymes in the ringtail possum would be uninduced in the ringtail. Pass *et al.* (In press) have shown that leaf terpenes induce CYP enzymes in the brushtail possum.

As reported earlier in this chapter (Section 6.2.1), gliders were necessarily maintained on a eucalypt leaf diet. In previous studies, gliders had shown a strong preference for *E. polycarpa*, and had thrived on a diet comprising this species alone (Dr W. J. Foley, personal communication). Such a preference suited the requirements of this study, as the gliders could be maintained on an essentially terpene free diet. However, on this occasion, gliders failed to thrive and leaf intake was reduced when only *E. polycarpa* was offered. Although not investigated, presumably significant seasonal or locational

variation in chemical composition of the leaves caused markedly different leaf palatability.

It was necessary to modify the diet to include a variety of eucalypt species before and after the experiment. Gliders were transferred to a pure *E. polycarpa* diet the day before the *p*-cymene dose which allowed a washout period for terpenes present in the mixed maintenance diet.

CHAPTER 7

P-CYMENE METABOLISM IN THE KOALA

7.1. Introduction

This fifth metabolism experiment completes the comparative study of *p*-cymene in the four folivores and the laboratory rat. In this experiment, the metabolism of the terpene, *p*-cymene, was studied in the ultimate specialist eucalypt folivore, the koala (*Phascolarctos cinereus*).

This study was performed in collaboration with Dr W. J. Foley and Ben Moore (PhD student), Division of Botany and Zoology, Australian National University, ACT.

This chapter describes both the procedure for dosing koalas with *p*-cymene and the methods for identifying and quantifying metabolites, including a proposed novel metabolite, and synthesising another.

7.2. Methods

7.2.1. Animals and Dosing

Six male koalas (weight 8.8 ± 1.0 kg (mean ± sd); identified as K 1 - 6) were captured on French Island, Victoria, Australia. They were housed at the Phillip Island Nature Park, Victoria and released back to their point of capture at the end of procedures. Koalas were held in purpose-built enclosures during their captivity and placed into metabolism cages (1.3 x 0.6 x 0.9 m high) within their enclosures for dosing experiments.

Koalas were given the same two oral doses of *p*-cymene used in other animals: 0.37 mmol/kg (50 mg/kg) and 1.49 mmol/kg (200 mg/kg). *p*-Cymene was combined in a paste of Portagen powder (an infant milk formula, Mead Johnson & Company, Evansville, Indianapolis, USA) and water and the koalas licked the dose from a syringe. *p*-Cymene concentration in the paste was 15 mg/g for the lower dose and 45 mg/g for the higher dose.

Urine and faeces were collected for 24 h prior to, and 2 x 24 h after, the administration of *p*-cymene. Urine was collected over dry ice and volume and pH measured. The floor of each metabolism cage was washed with distilled water and the washings combined with the days urine for metabolite analysis.

Faecal pellets were collected daily from a wire grate over the collecting funnel of the metabolism cages and weighed before freezing.

7.2.2. Leaf analysis

A leaf diet containing no, or minimal, *p*-cymene and preferably few other terpenes was required for feeding koalas during experiments. Leaf samples of available species of eucalypts (*E. cephalocarpa, E. globulus* and *E. ovata*) were analyzed for their terpene profile to identify the most appropriate diet. The method was similar to that used to analyse *E. polycarpa* in Chapter 6 - section 6.2.2. Leaf samples were also analysed by steam distillation as a comparison with the alcoholic extraction method (see Chapter 11 - section 11.2.2 for details on method).

Very low levels of *p*-cymene in *E. cephalocarpa* leaf extracts were detected by GC/MS using the selected ion monitoring (SIM) mode. *p*-Cymene concentrations were estimated as a relative proportion of the 1,8-cineole concentration, calculated by GC/MS analysis from the steam distillate of *E. cephalocarpa*. *p*-Cymene was found to be 0.3 % w/w of the 1,8-cineole concentration.

The 1,8-cineole concentrations of leaf collected from twelve trees were determined from a calibration curve and are reported in Chapter 11 - Table 11.1. The calibration curve was prepared from an ethanolic stock solution of 1,8-cineole (5 mg/ml) covering a concentration range of 0 - 2.5 mg/ml. Six dilutions of the 1,8-cineole stock solution were made and the volume made up to 0.5 ml with ethanol and 0.5 ml of internal standard stock solution (α-pinene; 3 mg/ml in ethanol).

In order to relate the abundances of the SIM *m/z*'s to the amount of each terpene, the selected *m/z*'s for *p*-cymene (119) and 1,8-cineole (154) were standardised as a fraction of their respective total ion current (TIC). The inverse of each fraction (2.8 and 35.7, respectively) was then multiplied by future SIM *m/z* abundances to calculate the TIC.

7.2.3. Urine analyses

Urine samples were diluted (1:2) and analyzed for both free and total metabolites and the levels of each major metabolite quantified. The method used is described in Chapter 2 - section 2.2.5. Chromatography resulted in the *p*-cymene related peaks being overwhelmed by leaf terpene metabolites (mainly 1,8-cineole metabolites; see Figure 7.1). It was therefore necessary to identify and quantify metabolites by extracting mass chromatograms of diagnostic ions from the TIC. Metabolites were identified by their GC retention times and mass spectra (see Table 7.1 and Figure 7.2). Mass spectral data for all metabolites, except Cy 13, have been published by others and were reported in Chapter 2 - Table 2.1 (Southwell *et al.* 1980; Ishida *et al.* 1981; Walde *et al.* 1983; Ishida *et al.* 1989).

Metabolites Cy 5 and 13 chromatographed poorly as their methyl ester derivatives and required further derivatisation of the hydroxyl groups with BSTFA to form the trimethylsilyl (TMS) derivatives. Therefore all samples were analyzed as both methylated only and methylated plus TMS derivatives. Methylated carboxylic acid metabolites were not affected by secondary TMS derivatisation. Nor did the secondary alcohols on the C8 position in Cy 2 and 13 form a TMS derivative as they were sterically hindered, however, the primary alcohol of Cy 5 and 13 was derivatised. The mass spectra and GC retention times of Cy 2, 6 and 10 were

unaltered by TMS derivatisation. Cy 10, the glycine conjugate of cumic acid, partially derivatised with BSTFA, accepting a TMS group on the nitrogen. Cy 10 could therefore only be analysed quantitatively as the methylated product.

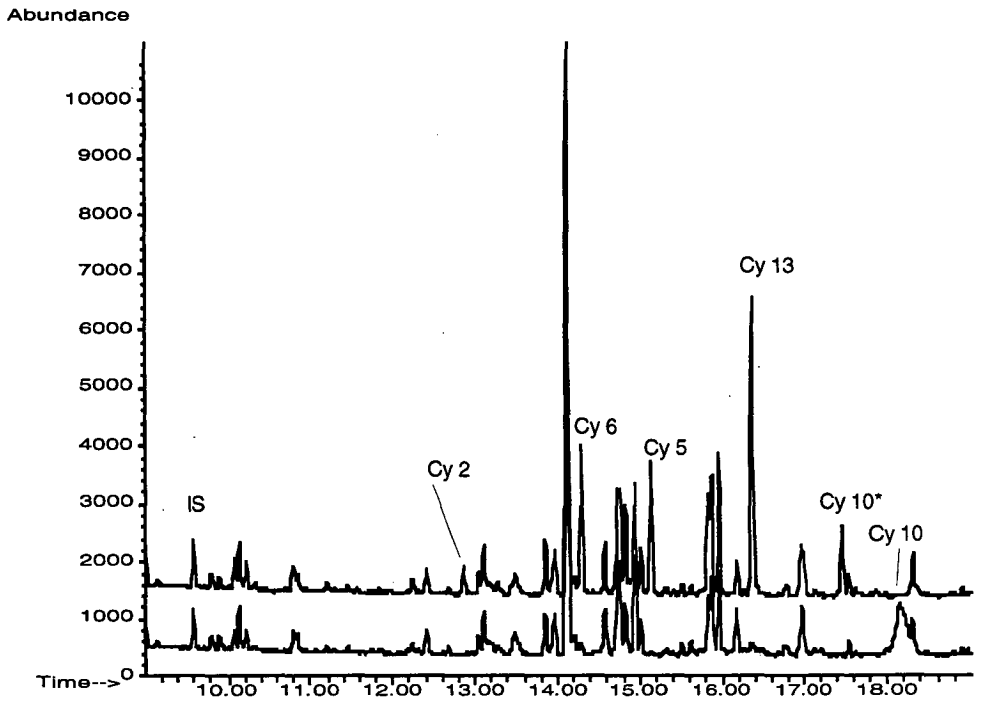


Figure 7.1. GC/MS chromatogram of hydrolysed koala urine extract for both a pre-dose (bottom chromatogram) and post-dose (top) sample. The urine extract was derivatised to the methylated and TMSed forms. *p* -Cymene metabolites are labelled and IS = internal standard (2,5-dimethyl benzoic acid methyl ester). Cy 10 was barely detectable after TMS derivatisation due to partial TMS derivatisation of the glycine moiety, resulting in Cy 10*. Therefore Cy 10 was quantified from a methylated only calibration curve. The urine sample was from Koala # 3, collected 24 - 48 h after the *p* -cymene dose of 1.49 mmol/kg.

Table 7.1. Assigned and chemical names of *p* -cymene metabolites excreted by the koala. Relevant data for the identification and quantitation of metabolites are also reported.

Metabolite	Chemical name	Derivative[1]	Rt (min) GC/MS	Quantified Y/N	Diagnostic ion at *m/z*	Purity[2] %
Cy 2	2-*p*- carboxyphenylpropan-2-ol	M	12.85	Y	179	81.2
Cy 5	2-*p*- carboxyphenylpropan-1-ol	M + TMS	15.14	Y	163	91.8
Cy 6	2-*p*- carboxyphenylpropionic acid	M	14.27	Y	163	95.3
Cy 10	*p*- isopropylbenzoylglycine (cuminuric acid)	M	17.86	Y	147	98.8
Cy 11	*p*- isopropenylbenzoylglycine	M	18.01	N	-	-
Cy 13	4-(1,2-dihydroxy-1-methylethyl)-benzoic acid	M + TMS	16.39	Y	179	-

[1]Methyl ester (M), trimethylsilyl ether (TMS)

[2]Purity of standards used for quantitation were estimated by GC analysis. Cy 13 was quantified using the Cy 5 (methyl ester and TMS derivative) calibration curve.

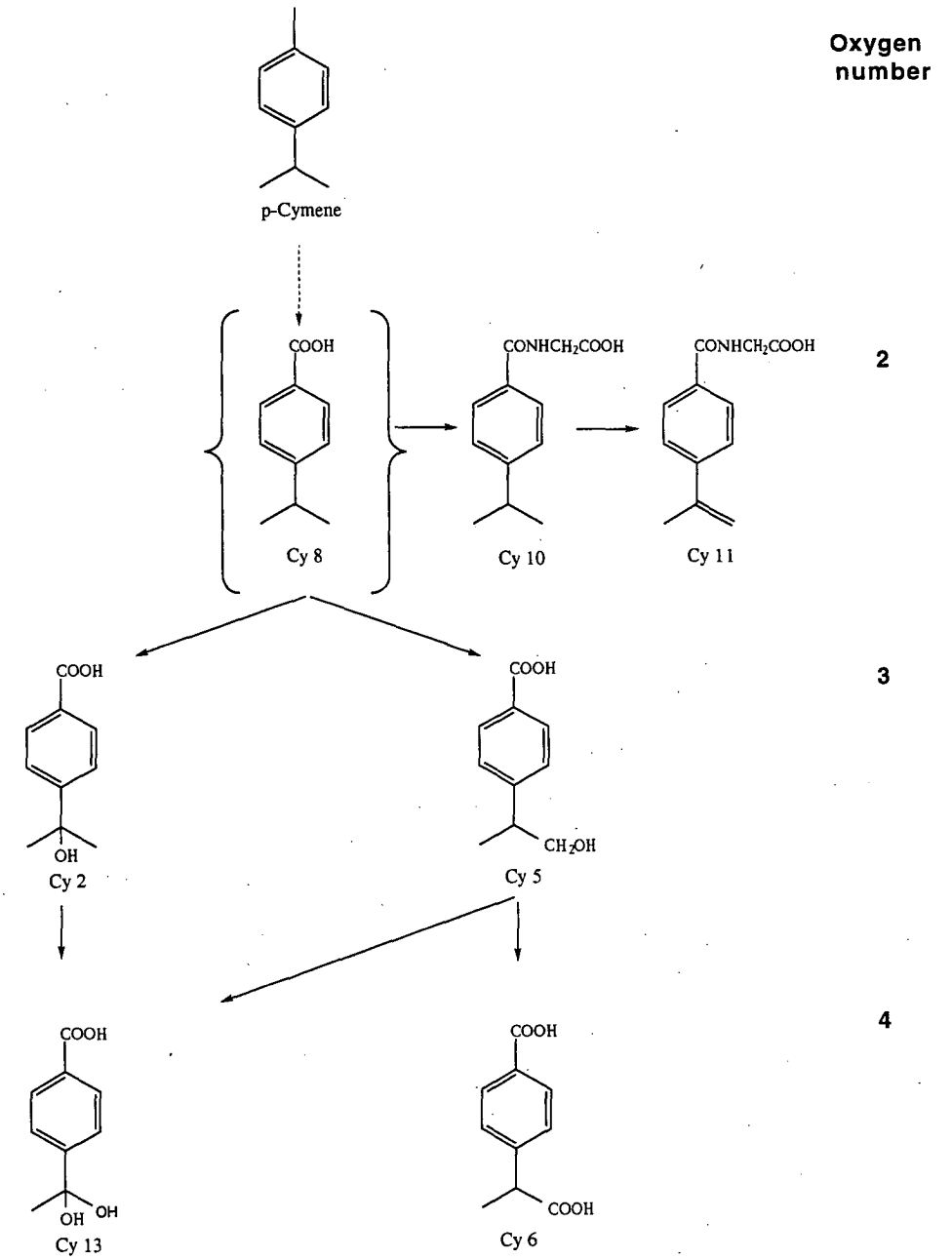


Figure 7.2. Chemical structures of *p* -cymene metabolites and probable metabolic pathway in the koala. Metabolites are shown in their underivatised forms. Dashed arrows indicate where intermediate metabolites have not been deteced. Cy8 was not detected in the koala but is a common precursor to all metabolites. Metabolites are grouped according to the number of oxygen atoms acquired and this is marked on the right hand side of the diagram.

Isolated metabolites Cy 2, 5 and 6 were available from previous work (Chapter 2). Metabolite Cy 13 was quantified using the calibration curve prepared for Cy 5, since their respective structures differed only in a hydroxyl group, which was sterically hindered and therefore did not accept a TMS group during derivatisation. Cy 10 was synthesized and the process is described below.

7.2.4. Synthesis of cuminuric acid (Cy 10)

Cuminuric acid (Cy 10) was synthesised from cumic acid in two stages. Initially the acid chloride was prepared and then subsequently reacted with glycine to produce the glycine conjugate, cuminuric acid. The method of forming the acid chloride and glycine conjugate were based on standard methods described in Vogel (1959) for the synthesis of benzoyl chloride and hippuric acid.

1) Synthesis of the acid chloride of cumic acid.

One gram of cumic acid was dried at 60°C under vacuum, transferred to a distilling flask and 1 ml (1.3 gm) redistilled thionyl chloride added. The flask was fitted with a condenser and a guard tube packed with cotton wool. The flask was then heated on a boiling water bath until the evolution of hydrogen chloride (HCl) ceased (approximately 1 h) (Figure 7.3 - Reaction 1).

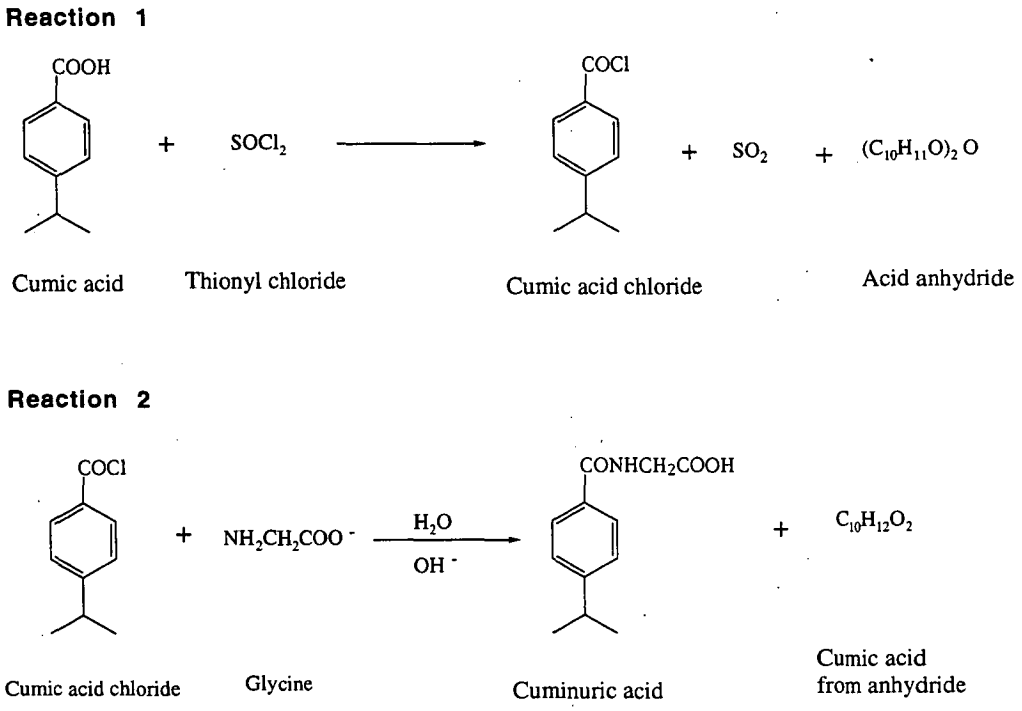


Figure 7.3. Synthesis of Cy 10 (cuminuric acid). Reaction 1 shows the formation of cumic acid chloride. Reaction 2 shows the formation of cuminuric acid.

2) Synthesis of cuminuric acid.

The Schotten-Bauman method of benzoylation was used to form an amide bond between the acid chloride and glycine (Figure 7.3 - Reaction 2). One gram of glycine was dissolved in 5 ml 10 % NaOH and transferred to a conical flask and chilled in ice. The cumic acid chloride mixture was added in two portions and shaken vigorously and returned to the ice. The mixture was then acidified with conc. HCl

(approximately 1 ml). The resulting crytalline precipitate was collected on a sintered glass filter and washed with water.

A portion of the crystals was dissolved in methanol and TLC performed to assess purity, using cumic acid as a standard. Pre-coated, aluminium backed silica gel 60 F_{254} (10 cm x 5 cm, DC-Alufolien, Kieselgel 60-F_{254}, Merck, Germany) plates were used and the mobile phase was ethyl acetate (100 %). All reagents and products, except glycine, quenched fluoresence and were therefore detectable by this method.

Impurities (Figure 7.4 - TLC-1) were removed from the crystals by further purification.

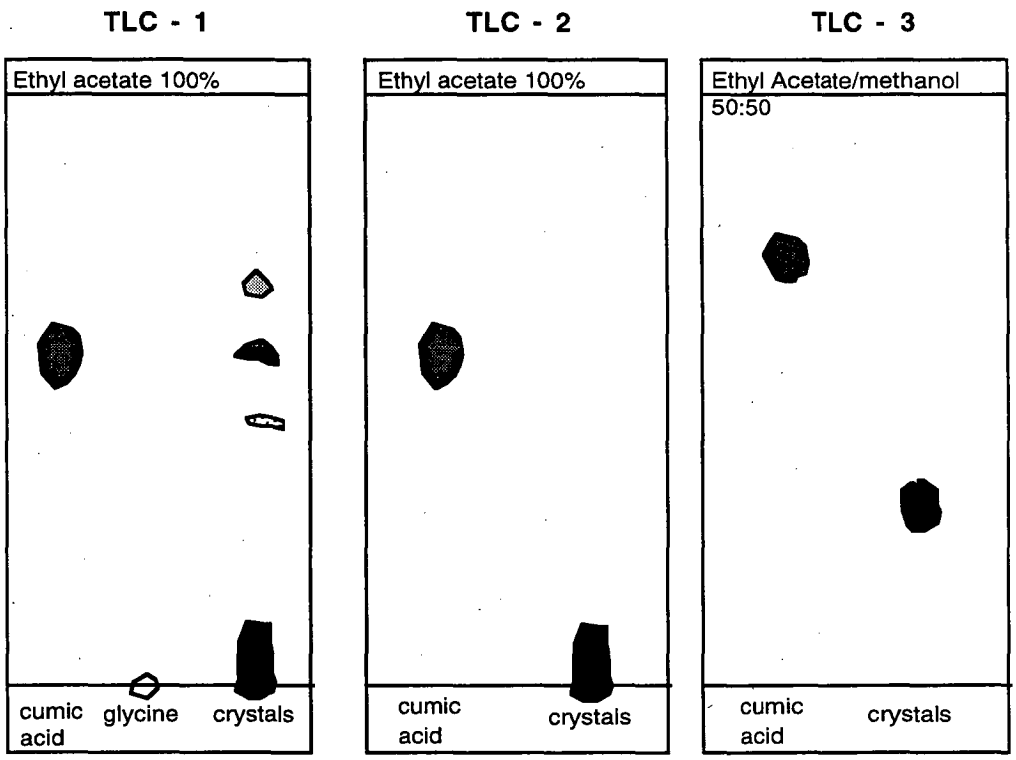


Figure 7.4. The synthesis of Cy 10 (cuminuric acid) was followed using TLC and UV fluorescence as a detector for a qualitative assessment of purity. Cumic acid was used as a reference on each plate. TLC-1 shows the raw product after synthesis. Note that glycine is not detected by UV fluorescence. A number of impurities were present and were successfully removed from the final crystals. TLC-2 and TLC-3 show the final crystals using two different mobile phases. The mass spectrum and GC/MS retention time were used to confirm the structure of Cy 10.

Approximately 25 % of the crystal yield was dissolved in ethyl acetate (30 ml) and glycine removed by 2 x 25 ml washes of 1 M HCl. Remnant acid was removed from the ethyl acetate by 3 x 25 ml washes of distilled water. Anhydrous sodium sulphate ($NaSO_4$) was added for 30 min to dry the ethyl acetate.

Cuminuric acid was then recrystallised from the ethyl acetate. Figure 7.4 shows the final product using ethyl acetate and ethyl acetate/methanol 50:50 as mobile phases respectively. The product appeared to be pure.

Crystals were then dried in a vacuum drying pistol at 60°C. Approximately 1 mg of cuminuric acid was placed into a test tube and dissolved in 1 ml ethyl acetate and methylated with diazomethane. Mass spectral data was consistent with that published by Walde *et al.* (1983) and was *m/z* (%): 235 (M^+, 10), 147 (100), 104 (7), 103 (7), 91 (12) and 77 (9). Purity of cuminuric acid (Cy 10) was confirmed by GC/MS to be 99 %.

7.2.5. Identification of novel metabolite (Cy 13)

GC/MS monitoring of an ion at *m/z* 179 (which was diagnostic for methylated Cy 2) disclosed an unfamiliar, major *p*-cymene metabolite at a significantly longer retention time to Cy 2. After additional derivatization (with BSTFA) to improve its chromatography, a tentative structure was deduced from the mass spectral data. Electron (EI) and chemical (CI) ionization and high resolution mass spectrometry provided evidence for the structure of this metabolite. EI mass spectra were obtained from the GC/MS instrument described in Chapter 2 - scetion 2.2.2. CI-GC/MS and high resolution MS analyses were carried out on a Hewlett-Packard model 5890 Series II GC directly coupled to a Kratos Concept ISQ high resolution mass spectrometer. For chemical ionisation, ammonia was used as the reagent gas, with an accelerating voltage of 5.3 kV, a scan rate of 0.9 sec/scan, *m/z* range of 100 - 550 and resolution of 1000 (3000 for accurate mass determinations). Perfluorokerosene (PFK) was used as an internal calibrant for accurate mass measurements.

7.2.6. Quantitation of urinary metabolites

Calibration curves were prepared for Cy 2, 5, 6 and 10 as described in Chapter 2 - section 2.2.5. The purity of standards used for quantitation were estimated by GC analysis and were reported in Table 7.1. Calibration curve parameters are detailed in Appendix 7 - Table A7.1 and were linear over the required concentration ranges.

7.2.7. Urinary glucuronic acid analysis

The total urinary glucuronic acid was measured for individual koalas before and after each dose of *p*-cymene. The method is described in Chapter 2 - section 2.2.8 and was based on that described by Blumenkrantz and Asboe-Hansen (1973). A two hundred-fold dilution with distilled water was suitable for most urine samples, however some required further dilution.

7.2.8. Faecal analysis

One pre- and two post-dose faecal samples from two koalas (K-2 and 6) were analyzed individually. The faecal pellets were ground in a mortar and pestle and weighed. A slurry was made from 5 g of each faecal sample and 20 ml of distilled

water. Weighed amounts (approximately 5 g) of each sample were placed into large centrifuge tubes. One sample was hydrolyzed to measure total metabolites by adding 5 ml of 1.1 M (pH 5.2) acetate buffer and 250 µl extract of *Helix Pomatia* (β-glucuronidase/arylsulphatase) and incubated overnight in a waterbath at 37°C. The second sample was analyzed for free metabolites only. After overnight incubation both samples were treated in the same manner. The pH was adjusted to 1 with 5 M HCl and the mixture extracted with three washes of ethyl acetate and then three washes of dichloromethane/n-propanol (80:20). The two solvent extractions were treated separately. Each extract was evaporated to dryness and redissolved in 2 ml ethyl acetate or 2 ml methanol for the ethyl acetate and dichloromethane/n-propanol extracts respectively. A sample of each of the final extracts was methylated with diazomethane and analyzed by GC/MS using the conditions described for urine analysis.

7.3. Results

7.3.1. Qualitative results

Six metabolites were identified in the urine of koalas (see Figure 7.2 and Table 7.1 for chemical structures and names). It is proposed that the major metabolite (Cy 13) is novel and it accounted for about half of the metabolites excreted (Table 7.2). It is an extensively oxidised compound having acquired a total of four oxygen atoms and has undergone oxidation at three different sites (Section 7.3.2).

The remaining five metabolites excreted by the koala have been identified in other animal species and reported in the literature (Southwell *et al.* 1980; Ishida *et al.* 1981; Walde *et al.* 1983; Matsumoto *et al.* 1992; Boyle *et al.* 1999).

7.3.2. Structural determination of Cy 13

Mass chromatograms of the ion at *m/z* 179 (see Figure 7.5 for structure) revealed two *p*-cymene metabolite peaks resulting from a β type cleavage of the aromatic ring occuring on the isopropyl group. The first peak was Cy 2, while the mass spectrum of the later eluting compound, Cy 13, had not previously been reported. EI GC/MS of Cy 13 as a methylated derivative revealed a fragmentation pattern similar to Cy 2. Two possible structures were initially considered for Cy 13, both structures differing from Cy 2 by having undergone oxygenation on a third site at C9. The first possibility was a dihydroxy acid (Cy 13) and the second a hydroxy diacid. CI GC/MS indicated a molecular weight of 210 from the intense ion at *m/z* 228 ($M+NH_4^+$) and EI GC/MS showed a trace of the M^+ ion at *m/z* 210. Cy 13 chromatographed poorly after methylation alone and the peak shape readily deteriorated. Chromatography improved dramatically after further derivatization with a TMS group, suggesting an additional hydroxy grouping. The C8 hydroxy group of Cy 13 (and Cy 2) did not accept the TMS group due to steric hindrance.

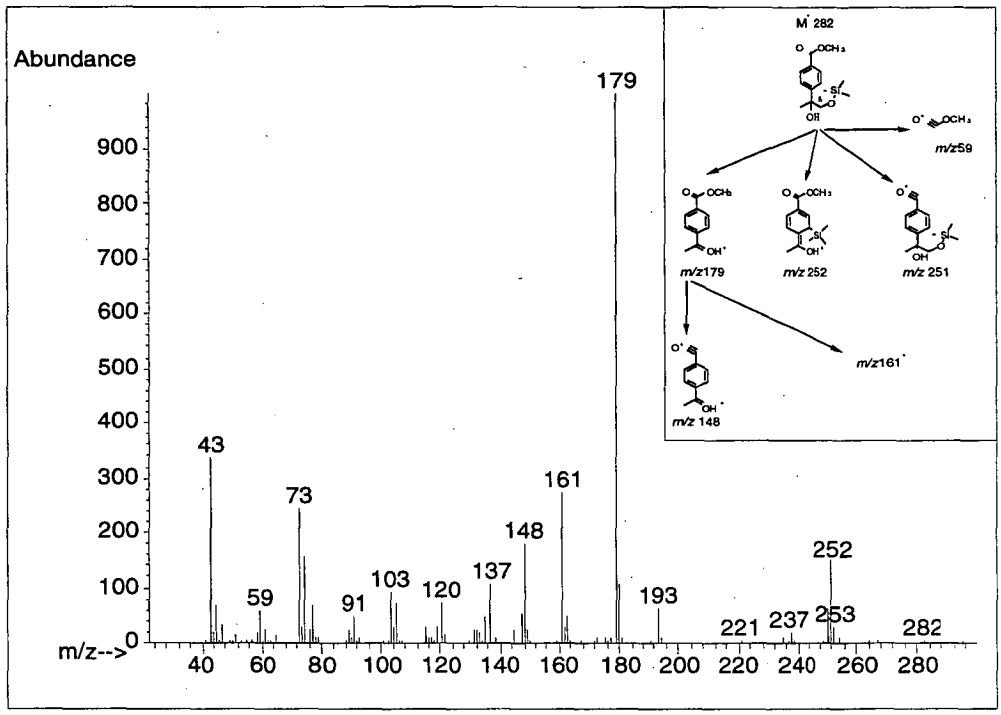


Figure 7.5. Mass spectrum of Cy 13 (methyl ester and TMS derivative). Inset: Proposed fragmentation pattern of Cy 13 (methyl ester and TMS derivative) as depicted in the mass spectrum. The rearrangement of silicon onto the aromatic ring results in an ion at M^+-30 (*m/z* 252).

The EI mass spectrum of Cy 13 (methyl ester and TMS derivative) was *m/z* (%): 282 (282.1229, $C_{14}H_{22}SiO_4$ calculated mass 282.1287, M^+(trace)), 252 (252.1173, $C_{13}H_{20}SiO_3$ calculated mass 252.1182, M^+-30 (18)), 251 (M-OCH_3, (9)), 179 (179.0717, $C_{10}H_{11}SiO_3$ calculated mass 179.0708, M^+-103 (100)), 161 (24), 148 (16), 137(9), 120 (8), 105 (8), 103 (9), 91 (5), 77 (8), 75 (23), 73 (36), 59 (7) and 43 (41) (see Figure 7.5). The elimination of CH_2O (M^+-30) from the molecular ion requires intramolecular rearrangement involving the TMS group. The rearrangement and resulting fragmentation pattern are illustrated in Figure 7.5 (Inset). The methyl ester of an analagous compound, α-[[(trimethylsilyl)oxy]methyl] benzene acetic acid, undergoes the same rearrangement providing a model for this type of rearrangement (NIST 1998).

The metabolite Cy 5 also has a primary alcohol at the C9 position. It would therefore be expected that the molecular ion of its TMS derivative would undergo similar rearrangement and fragmentation as Cy 13 (methyl ester and TMS derivative) and α-[[(trimethylsilyl)oxy]methyl] benzene acetic acid (methyl ester). Again the mass spectral data confirmed this pattern, although no molecular ion at 266 was detected for Cy 5. The EI mass spectra of Cy 5 (methyl ester and TMS derivative) was *m/z* (%): 251 (26), 237 (12), 236 (236.1257, $C_{10}H_{11}SiO_3$ calculated mass 236.1233, M-CH_2O (61)), 235 (10), 132 (46), 104 (14), 103 (41), 91 (10), 77 (14), 75 (22), 74 (11), 73 (100), 59 (12) and 45 (14).

The combined mass spectral data provides strong evidence for the structure of Cy 13 being 4-(1,2-dihydroxy-1-methylethyl)-benzoic acid. Ishida *et al.* (1982) tentatively assigned this same structure to a metabolite of perilla aldehyde based on EI mass spectral data alone. However, their EI data does not support the structure now assigned to Cy 13, which is based on considerably more extensive MS data and the fragmentation behaviour of related model compounds. It is therefore proposed that this is a novel metabolite of *p*-cymene.

7.3.3. Quantitative results

Metabolites Cy 2, 5, 6, 10 and 13 were all present in amounts sufficient to quantify. Cy 11 was detected as a trace metabolite and not quantified. Table 7.1 records the *m/z*'s used for extracting mass chromatograms from the TIC to identify and quantify metabolites by GC/MS as well as listing the chemical names of metabolites, derivatization required for chromatography and the purity of isolated metabolites used as standards for quantitation.

The fraction of the administered dose of 0.37 mmol/kg *p*-cymene recovered in 48 h as free and total was, respectively, 0.82 ± 0.08 and 0.77 ± 0.09 (mean ± sd; n = 5). The fraction recovered for the dose of 1.49 mmol/kg was 0.77 ± 0.14 and 0.84 ± 0.12 for free and total metabolites respectively (n = 6).

Most of the recovered metabolites were excreted in the first 24 h (median 77% for both doses and ranges 0 – 97% for the lower dose and 0 – 100% for the higher dose). Koala #1 produced no urine for 24 h after each dose and was responsible for the large range in the 24 h recovery of metabolites. Results from the two 24 h post-dose urine collections were combined.

Figure 7.6 depicts pictorially the pattern of metabolite excretion and reports the molar recovery of each metabolite per body weight (kg^{-1}). There was no significant increase in the recovery of any individual metabolite, after hydrolysis, suggesting there was no significant hydrolysable conjugation of *p*-cymene metabolites (Figure 7.6).

The excretion of free, conjugated and total metabolites, expressed as the percentage of total recovered metabolites, are reported in Table 7.2.

Clearly, the novel metabolite, Cy 13, was the most abundant metabolite. The second most important metabolite, the dicarboxylic acid, Cy 6, acquired the same number of oxygen atoms.

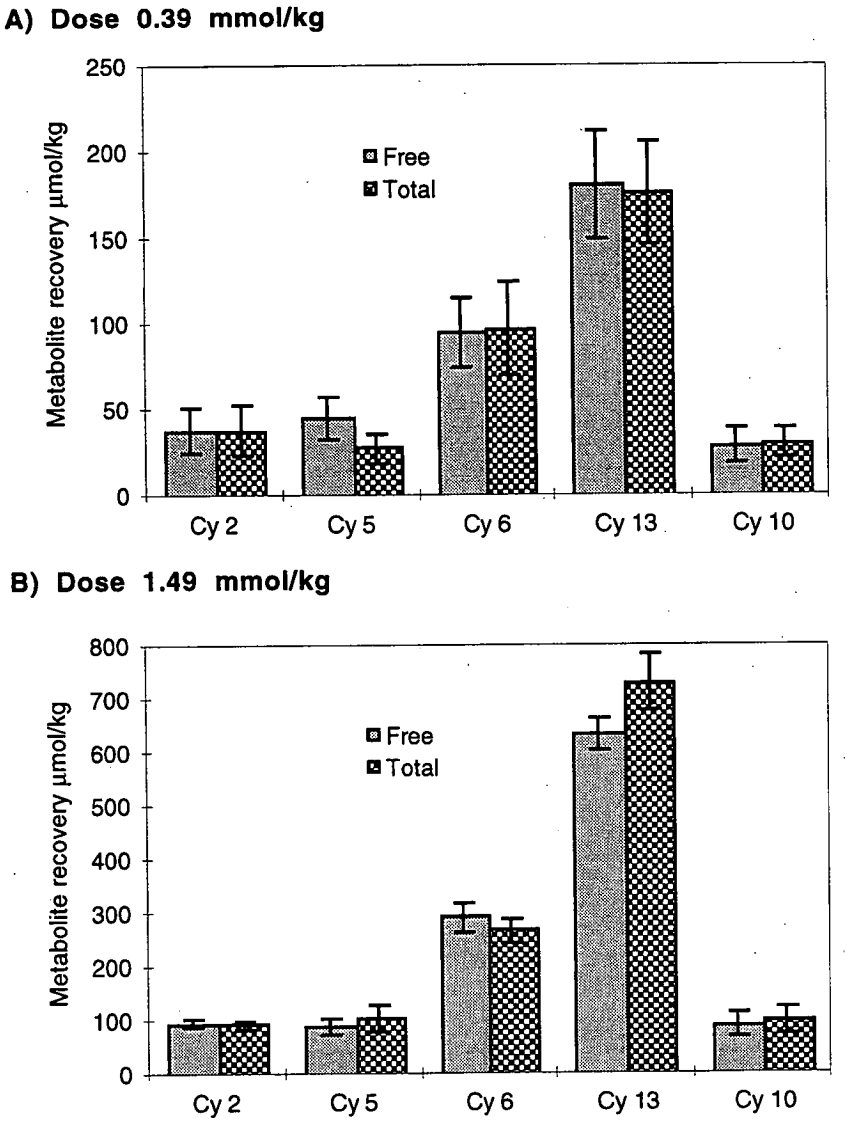


Figure 7.6. Molar recoveries (mean ± se) of free and total levels of metabolites of *p* -cymene in the koala. Hydrolysis had no effect on metabolite recovery.

Table 7.2. Excretion of free, conjugated and total metabolites expressed as a percentage of total recovered metabolites after both doses of *p* -cymene in the koala (n = 6).

	Percent (mean ± SD) of total urinary metabolites					
	Dose 0.37 mmol/kg			Dose 1.47 mmol/kg		
Metabolite	Free	Conjugated	Total	Free	Conjugated	Total
Cy 2	9.5 ± 4.7	0.5 ± 0.7	9.1 ± 3.7	7.6 ± 2.2	0.2 ± 0.3	7.3 ± 1.8
Cy 5	12.2 ± 5.8	1.3 ± 2.7	7.2 ± 3.3	7.3 ± 3.6	1.6 ± 2.5	8.4 ± 5.1
Cy 6	26.1 ± 3.4	2.4 ± 2.3	26.0 ± 7.0	22.9 ± 5.7	0.6 ± 1.2	20.7 ± 3.0
Cy 10	7.4 ± 4.8	0.9 ± 1.5	8.0 ± 3.8	6.7 ± 3.4	1.0 ± 0.7	7.3 ± 3.6
Cy 11	tr	tr	tr	tr	tr	tr
Cy 13	50.9 ± 6.9	1.8 ± 2.7	49.7 ± 7.6	49.6 ± 8.4	7.3 ± 8.5	56.3 ± 5.2
Sum	106.1 ± 5.6	0	100	94.2 ± 15.4	9.6 ± 11.2	100

Comparison of free and total metabolite recovery within dose and between doses, $P > 0.05$ (Student's paired *t* -test).
Each value is the mean ± sd of individual koalas. Free and total metabolites were determined, respectively, before and after hydrolysis, and the difference was considered to be due to the conjugated metabolites. For some metabolites free levels were slightly greater than total, and the value of the difference was considered to be "0". Fractional recoveries of *p* -cymene were 0.82 ± 0.08 and 0.77 ± 0.09 for the low and high dose respectively.

Metabolites were grouped according to the number of oxygen atoms acquired during oxidation (Figure 7.7). Together, Cy 6 and 13 (four oxygens) accounted for the majority (75 %) of the recovered dose. Cy 2 and 5 (3 oxygens) were also combined and Cy 10 (and Cy 11), which are glycine conjugates of the Cy 8, were considered to have gained two oxygens by oxidation.

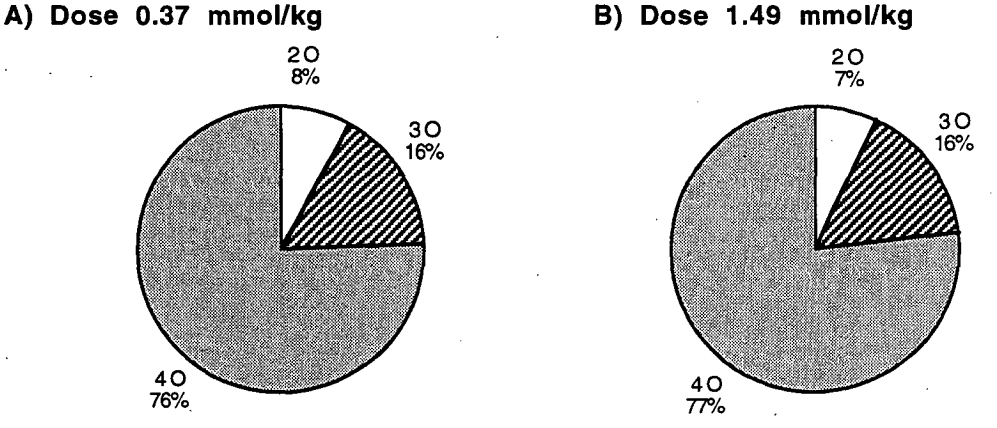


Figure 7.7. Metabolites grouped according to the number of oxygen (O) atoms acquired during oxidation and reported as the percent of recovered dose after hydrolysis for **A)** 0.37 mmol/kg and **B)** 1.49 mmol/kg of *p* -cymene.

7.3.4. Leaf analysis

The terpene profiles of three readily available *Eucalyptus* species were analysed for their suitability as a diet for dosing experiments. *E. globulus* leaf extract contained only trace amounts of *p*-cymene and large quantities of 1,8-cineole, however, it also contained a large number of sesquiterpene compounds as well as α-pinene. *E. ovata* also contained trace amounts of *p*-cymene plus large quantities of linalool (but no 1,8-cineole). There were also a large number of sesquiterpenes. *E. cephalocarpa* leaf extracts contained no sesquiterpenes and the major monoterpene was 1,8-cineole (see Chapter 11 - Figure 11.1 for the GC/MS of *E. cephalocarpa* leaf extract). There were minor amounts of limonene, α-pinene and *p*-cymene. *E. cephalocarpa* was chosen as the most suitable diet due to the simplicity of the terpene profile, minimising interference in urine metabolite analyses from leaf derived PSMs. An added advantage of this choice was the opportunity to study the metabolic fate of 1,8-cineole in the koala after chronic ingestion (Chapter 11).

The daily consumption of *E. cephalocarpa* leaf and estimated intakes of 1,8-cineole and *p*-cymene are recorded in Table 7.3. The daily intake of *p*-cymene from leaf was approximately 10 mg/day (0.075 mmoles/day) and was considered to be negligible compared to the *p*-cymene dose (0.37 and 1.49 mmol/kg).

Table 7.3. Leaf ingested (dry mass = DM),1,8-cineole and background *p* -cymene intake for each day of both *p* -cymene dosing experiments.

Koala	Experiment	DM leaf eaten (g)		1,8-Cineole eaten (g)[2]		*p* -Cymene intake (mg)[3]	
	day	Dose 1[1]	Dose 2[1]	Dose 1[1]	Dose 2[1]	Dose 1	Dose 2
1	0	149	204	4.1	3.3	11	9
1	1	131	136	3.5	3.2	10	9
1	2	123	129	3.3	3.0	9	8
2	0	115	187	3.5	3.1	10	8
2	1	116	139	3.9	2.7	11	7
2	2	131	83	4.4	1.7	12	4
3	0	126	215	3.8	2.6	10	7
3	1	112	154	3.4	2.3	9	6
3	2	116	112	3.5	1.7	9	5
4	0	162	193	5.2	5.4	14	15
4	1	161	170	5.2	4.7	14	13
4	2	176	181	5.7	5.0	15	14
5	0	243	292	4.3	6.3	12	17
5	1	185	191	3.8	4.1	10	11
5	2	178	188	3.7	4.0	10	11
6	0	137	184	2.4	6.9	7	19
6	1	136	124	2.2	4.6	6	13
6	2	128	92	2.0	3.4	6	9
	Mean	146	165	3.8	3.8	10	10
	sd	34	50	1.0	1.5	3	4

[1]Dose 1 = 0.37 mmol/kg and dose 2 = 1.49 mmol/kg. In each experiment *p* -cymene was administered on day 1.
[2]1,8-Cineole concentrations were measured directly in the leaf and 1,8-cineole intake calculated from amount of leaf ingested. These values were used in Chapter 11 in the study of the metabolic fate of 1,8-cineole in the koala.
[3]*p* -Cymene intake was estimated on the basis of 1,8-cineole concentrations. Abundance of *p* -cymene was found to be 0.3 % of 1,8-cineole.

7.3.5. Glucuronic acid

Table 7.4 reports the measured levels of glucuronic acid for each koala throughout both dosing experiments. Although the koalas excreted large quantities of glucuronic acid daily, there was no significant increase in glucuronic acid excretion after *p*-cymene was administered.

Table 7.4. Urinary glucuronic excretion (µmol/kg) after each dose of *p* -cymene in individual koalas.

	Urinary glucuronic acid (µmol/kg)					
	Dose 0.37 mmol/kg[1]			Dose 1.49 mmol/kg[1]		
Koala	Day 0[2]	Day 1	Day 2	Day 0[2]	Day 1	Day 2
1	2387	0[3]	1820	1296	0[3]	1650
2	971	899	397	554	636	651
3	685	489	774	716	534	491
3	0[3]	1449	1378	1484	1039	957
5	704	3405	1435	1126	386	2491
6	693	1358	1211	1284	918	645
Mean	1088	1520	1169	1077	703	1148
sd	736	1122	508	364	271	778

[1]*p* -Cymene dose.

[2]Day 0 is the pre-dose control, collected for 24 h.

[3]no urine voided.

There was no significant difference in glucuronic acid between days for each dose (Anova (single factor) for 0.37mmol/kg; *df* = 2, F = 0.76, *P* = 0.76 and 1.49mmol/kg; *df* = 2, F = 1.92, *P* = 0.18).

nb. 1000 µmole glucuronic acid = 194 mg

7.3.6. Faecal analysis

GC/MS analysis of faecal extracts revealed no unchanged *p*-cymene or oxidised metabolites, or their glucuronides. Unchanged 1,8-cineole was detected in small amounts in some koalas.

7.3.7. Animal details

Urine volumes for 0.37 and 1.49 mmol/kg *p*-cymene were 106 ± 64 ml and 92 ± 46 ml (mean ± sd) respectively. Anova (single factor) comparing pre- and post-dose urine output and pH indicated that the *p*-cymene doses had no affect on these parameters. pH measurements were 5.7 ± 0.2 and 5.5 ± 0.3 (mean ± sd) throughout dose 1 and 2, respectively (Appendix 7 - Table A7.3). Koala urine remained acidic throughout, reflecting the excretion of acidic, leaf derived, metabolites.

Molar recoveries of each metabolite are reported in Appendix 7 - Table A7.2.

7.4. Discussion

The results of this study demonstrate that the koala employs a metabolic strategy in the detoxification of *p*-cymene which promotes rapid production and elimination of extensively oxidised metabolites. The koala excreted six metabolites of *p*-cymene, all of which were either extensively oxidised at multiple sites (Cy 13, 6, 5 and 2) or oxidised to a precursor carboxylic acid (cumic acid) and then conjugated with the amino acid glycine (Cy 10 and 11). No significant conjugation of metabolites with glucuronic acid was detected. Nor did the urinary glucuronic acid concentrations indicate conjugation of *p*-cymene metabolites (Table 7.4). However, the large standard deviations in glucuronic acid concentrations suggest that these determinations would be insensitive to minor conjugation of *p*-cymene metabolites.

The absence of glucuronidation of *p*-cymene metabolites is noteworthy considering the large amount of glucuronic acid found in the urine. The koalas excreted about 2 g of glucuronic acid daily (Table 7.4) suggesting that glucuronidation does have an important role in the elimination of some PSM component of *E. cephalocarpa.* This has been given further attention in Chapters 9 and 11.

The pattern of metabolite excretion was comparable between the low and high doses of *p*-cymene. This suggests that there was no saturation of detoxification enzymes at these dosage levels of *p*-cymene. The dosage regimen administered to the koalas, compared to the amount of leaf 1,8-cineole ingested (see Table 7.3), suggests that the koala could tolerate very much larger doses of *p*-cymene.

Examining the chemical structures of metabolites provides information on the enzymatic pathways involved in detoxification (Figure 7.2). All six metabolites underwent preliminary oxidation at C7 to form cumic acid. Although cumic acid was not excreted by the koala, it is a significant urinary metabolite in the rat, brushtail possum and rabbit (Ishida *et al.* 1981; Walde *et al.* 1983; Boyle *et al.* 1999). In the koala about 7% of the cumic acid was conjugated with glycine while the majority underwent further oxidation at one or more sites. The majority of the cumic acid excreted by the rat and brushtail was conjugated with glucuronic acid (Boyle et al., 1999) and none with glycine. It would seem cumic acid is not excreted in these species without further alteration to a more polar compound.

The glycine-conjugated metabolites, Cy 10 and 11, accounted for 7 - 8 % of the recovered metabolites in the koala. Southwell (1974) also reported that cumic acid conjugated with glycine to form cuminuric acid in the urine of koalas fed *E. punctata.* Yet, the glycine metabolites were not detected at all in the ringtail or glider, despite the glycine conjugation pathway being active in all species since hippuric acid, the glycine conjugate of benzoic acid, was excreted in each case.

The C7 position of *p*-cymene was the most reactive site for enzymatic oxidation by both the CYP and dehydrogenase enzymes since all metabolites had a carboxylic acid moiety on this position before conjugation. Oxidation also occurred at the C8 and C9 positions. Simple hydroxylation at each of these positions produced Cy 2 and 5 respectively. Final oxidations resulted in Cy 13 (a second hydroxylation on C9) and Cy 6 (oxidation of the C9 alcohol to a carboxyl group). Cy 6 has been reported as a common *p*-cymene metabolite in other animal species and accounted for about 25%

of the recovered dose in the koala. These two metabolites combined accounted for the majority of the metabolites recovered.

Cy 13 was found, despite its poor chromatography, because of its abundance in koala urine. Using the double derivatization method developed here, urine samples from the other animal species used in our previous studies were reanalyzed (Boyle *et al.* 1999). Cy 13 was detected in all species (rat, brushtail possum, ringtail possum and greater glider) and although not quantified was present in minor amounts, compared to the abundance of other metabolites.

The recovery of the *p*-cymene doses (approximately 80 %) was satisfactory. Natural variability in koala behaviour, in the ingested dose and experimental procedures may account for some of the remaining 20 %, as well as other factors which are discussed in Chapter 8 - section 8.3.4.

Wild animals are difficult to work with and must be handled and housed carefully to minimize stress to the animals. Administering an oral dose to any animal is stressful, particularly with standard procedures such as gavaging. It should be noted the method devised for dosing koalas in this experiment appeared to cause very little stress in the animals. The koalas actively participated in the dosing procedure, licking the dose from a syringe. Accuracy of the dose received was acceptable, with only an occasional messy licker, after which the koala would lick the paste containing the *p*-cymene from its fur.

CHAPTER 8

INTER-SPECIES COMPARISON OF P-CYMENE METABOLISM

8.1. Introduction

In this chapter, the results from the previous five chapters have been combined to allow comparisons of *p*-cymene metabolism between each animal species studied. The results are considered from an ecological perspective. An understanding of *p*-cymene metabolism provides an insight into the strategies employed which allow these animals to cope with a diet containing high concentrations of these types of compounds.

8.2. Results

The major findings for each species are compiled, summarised and reported in this section. Results are expressed as either molar recoveries standardised for animal weight (kg^{-1}) or percentage of recovered dose (or excreted metabolites). This enabled direct comparisons between species of widely differing weights (0.2 - 8 kg).

8.2.1. Qualitative results

A summary of the recovery data for all metabolites identified in this series of experiments is shown in Figure 8.1. The chemical name for each compound was reported in Chapter 2 - Table 2.1.

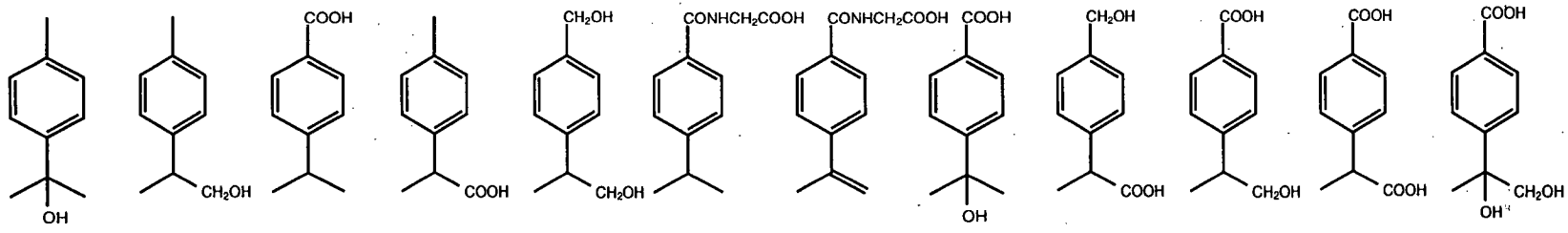
The metabolism of *p*-cymene was complex and varied markedly between species. Both the range of oxidised metabolites excreted and the role of glucuronidation varied between species. Only the rats and brushtail possums showed significant glucuronidation of *p*-cymene metabolites. Small amounts of glycine conjugates were also detected in some species and not in others. Table 8.1 and Figure 8.1 show the range of metabolites excreted by each species.

8.2.2. Quantitative results

8.2.2.1. Total metabolite recovery

Table 8.2 summarises the fraction of the dose recovered as urinary metabolites for each species. The percent of urinary metabolites which were excreted in the first 24 h was expressed as the median due to the large variability of recoveries within species and is shown in Table 8.3.

Table 8.1. Urinary excretion of *p* -cymene metabolites in each species, measured after hydrolysis of glucuronides. Metabolites are listed in approximate order of increasing polarity (left to right) based on the number of oxygen atoms acquired prior to conjugation.



Number of oxygen atoms acquired	1	1	2	2	2	2	2	3	3	3	4	4

Increasing degree of oxidation →

Species	N	Dose (mmol/kg)	Cy 1	Cy 4	Cy 8	Cy 9	Cy 12	Cy 10*	Cy 11*	Cy 2	Cy 3	Cy 5	Cy 6	Cy 13
			Percent (mean ± sd) of total urinary metabolites excreted in 48 h											
R	6	0.37	39.2 ± 2.5	tr	16.7 ± 2.5	-	tr	tr	-	19.5 ± 2.7	tr	10.3 ± 2.6	14.3 ± 2.8	†
	5	1.49	34.6 ± 3.6	tr	18.6 ± 3.5	-	tr	tr	-	23.3 ± 1.1	tr	9.7 ± 2.0	13.7 ± 1.2	†
BP	6	0.37	-	tr	6.6 ± 5.8	13.8 ± 2.9	-	tr	tr	4.4 ± 2.3	-	56.8 ± 5.4	18.4 ± 3.8	†
	6	1.49	-	tr	16.9 ± 10.3	9.5 ± 2.8	-	tr	tr	8.0 ± 1.8	-	59.0 ± 9.7	6.6 ± 1.9	†
RT	3	0.37	-	-	-	-	-	-	-	36.3 ± 3.8	22.6 ± 2.5	tr	41.0 ± 2.6	†
GG	6	1.49	-	-	-	-	-	-	-	12.1 ± 2.1	-	42.0 ± 2.5	45.9 ± 4.1	†
K	6	0.37	-	-	-	-	-	8.0 ± 3.8	tr	9.1 ± 3.7	-	7.2 ± 3.3	26.0 ± 7.0	49.7 ± 7.6
	6	1.49	-	-	-	-	-	7.3 ± 3.6	tr	7.3 ± 1.8	-	8.1 ± 4.5	20.7 ± 3.0	56.3 ± 5.2

R = rat, BP = brushtail possum, RT = ringtail possum, GG = greater glider, K = koala, tr = trace (< 4 %, but not quantified), - = not detected.

*Cy 10 and 11 are glycine conjugates of 2-oxygen metabolites.

†Cy 13 was found retrospectively in urine extracts of these species. Comparison of peak height to other metabolites suggests that Cy 13 could account for up to 2 or 3 percent of the recovered metabolites.

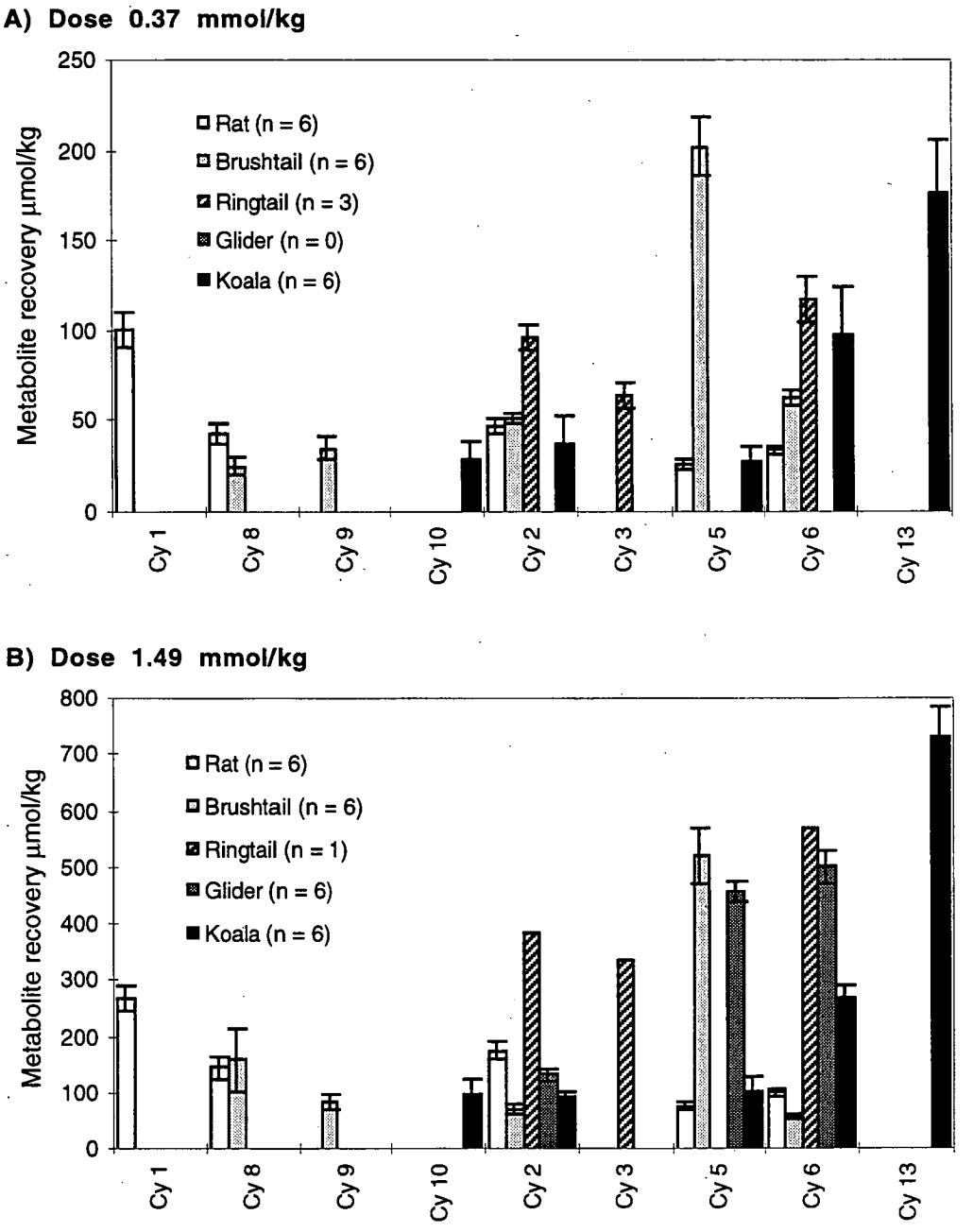


Figure 8.1. Total metabolite recoveries (µmol/kg body weight; mean ± se) of individual metabolites excreted by each species after *p* -cymene. Metabolites are shown in approximate order of degree of oxidation (left to right).

Table 8.2. Fractional recovery in 48 h of the administered dose of *p* -cymene in each species studied.

	Fractional recovery of administered dose (mean ± sd)	
Species	Dose 0.37 mmol/kg	Dose 1.49 mmol/kg
rat	0.65 ± 0.12 (n = 6)	0.52 ± 0.09 (n = 6)
brushtail	0.62 ± 0.17 (n = 6)	0.60 ± 0.14 (n = 6)
ringtail	0.74 ± 0.07 (n = 3)	0.87 (n = 1)
glider	-	0.74 ± 0.07 (n = 6)
koala	0.77 ± 0.09 (n = 6)	0.84 ± 0.12 (n = 6)

Table 8.3. Percent of recovered dose excreted in the first 24 h.

	Percent of recovered dose excreted in the first 24 h, median (range)[1]	
Species	Dose 0.37 mmol/kg	Dose 1.49 mmol/kg
rat	99 (93 - 100)	96 (90 - 100)
brushtail	15 (0 - 81)	88 (12 - 92)
ringtail	81 (45 - 97)	97
glider	-	95 (84 - 100)
koala	77 (0 - 97)	77 (0 - 100)

[1]Median (range) values are reported due to the inter-animal variation in 24 h recoveries.

Table 8.1 reports the metabolite excretion pattern, in each species, as the percentage of total urinary metabolites found. Metabolites are listed in approximate order of degree of oxidation, from least to most oxidised, based on the number of oxygen atoms gained through oxidative metabolism. Figure 8.1 shows graphically the molar amount of each metabolite excreted in each species, and also lists metabolites from least to most oxidised.

The pattern of metabolite excretion reflected the occurrence of eucalypt leaves, and hence terpenes in general, and *p*-cymene in particular, in the diet of each species. Figure 8.2 depicts the adaptation of detoxification mechanisms from the omnivorous rat, the generalist brushtail possum and through the spectrum of specialist folivores, by grouping metabolites according to the degree of oxidation undergone.

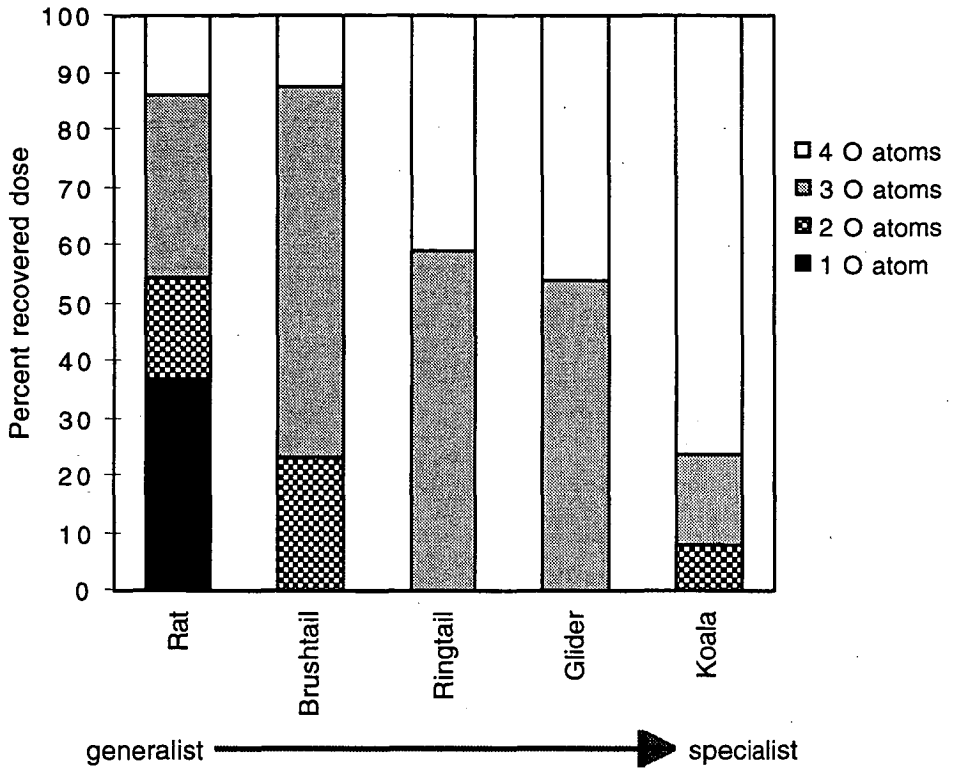


Figure 8.2. Comparative oxidation of *p* -cymene in generalist and specialist eucalypt folivores and the eutherian rat. Metabolites are categorised by the number of oxygen atoms acquired. Where two doses were administered, data were averaged. Note that the 2-oxygen metabolites in the koala were conjugated with glycine, preventing further oxidation.

8.2.2.2. Metabolite conjugation

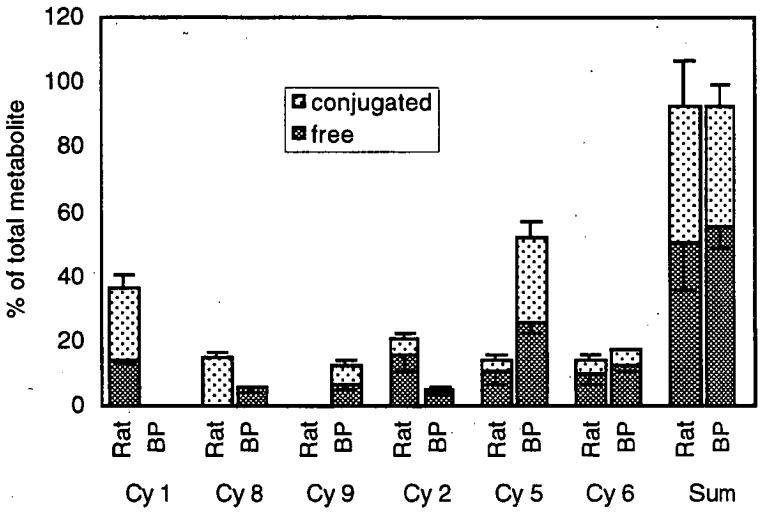
Conjugation was detected with both glucuronic acid and glycine. Glucuronidation was significant only in the rat and brushtail possum. Figure 8.4 shows the metabolites conjugated and degree, measured in the rat and brushtail possum.

Traces of the glycine conjugated metabolites, Cy 10 and 11, were detected in the rat and brushtail possum. Significant amounts of Cy 10 were measured in the koala.

8.2.2.3. Urinary glucuronic acid

The total urinary excretion of glucuronic acid by each species is summarised in Table 8.4.

A) 0.37 mmol/kg



B) 1.49 mmol/kg

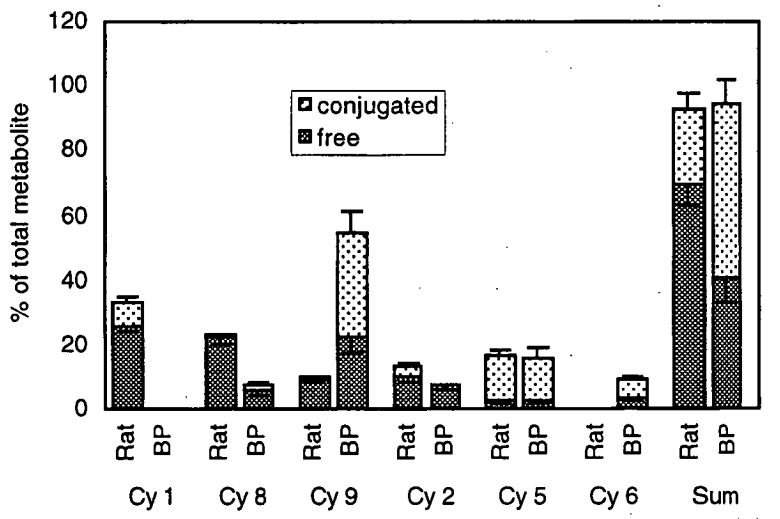


Figure 8.3. Total and conjugated p-cymene metabolites excreted by the rat and brushtail possum (BP) expressed as percentage of total metabolites (mean ± se). Metabolites are in approximate order of increasing degree of oxidation (left to right).

Table 8.4. Comparison of glucuronic acid excretion between species.

Species (Dose)	Diet	n	Day 0 (pre-dose)	Day 1	Day 2	*P* -value[1]
			µmol/kg (mean ± sd)			
Rat						
0.37 mmol/kg	artificial	4	159 ± 80	269 ± 130	144 ± 65	<0.05
1.49 mmol/kg	artificial	6	185 ± 51	675 ± 111	280 ± 92	<0.05
Brushtail possum[2]						
0.37 mmol/kg	artificial	6	93 ± 198	60 ± 47	34 ± 48	
1.49 mmol/kg	artificial	6	-	550 ± 293	151 ± 247	
Ringtail possum						
0.37 mmol/kg	artificial	3	42 ± 35	73 ± 111	1.7 ± 2.1	>0.05
Greater glider[3]						
1.49 mmol/kg	*Eucalyptus* leaf	*6*	-	2594 ± 1999		>0.05
Koala						
0.37 mmol/kg	*Eucalyptus* leaf	*6*	1091 ± 742	1519 ± 1120	1174 ± 515	>0.05
1.49 mmol/kg	*Eucalyptus* leaf	*6*	1079 ± 362	703 ± 268	1151 ± 779	>0.05

[1]Comparing pre- and post - dose (day 1) glucuronic acid measurements.
[2]Brushtail possum pre-dose glucuronic acid measurements were not a full 24 h period, therefore a direct comparison between pre- and post dose concentrations could not be made.
[3]Glider pre-dose glucuronic acid measurements were not a full 24 h period, so glucuronic acid levels were standardised with creatinine. Therefore a pre-dose value is not reported and the post-dose value is the average of the post-dose measurements for day 1 and 2. The *P* -value was calculated from the glucuronic acid/creatinine ratio.

8.3. Discussion

8.3.1. Excretion patterns

Combining the results from the studies in Chapters 3 to 7 provides a valuable insight into the comparative detoxification mechanisms employed by all marsupial eucalypt leaf eaters, specialists (ringtail possum, greater glider and the koala) and generalist (brushtail possums). The rat provided a comparison with a eutherian generalist.

Specialist and generalist herbivores had markedly different patterns of excretion of *p*-cymene metabolites. Three different patterns of metabolism were evident. First, metabolites excreted by the specialists were all extensively oxidised (with the addition of at least three oxygen atoms), whereas the generalists excreted metabolites with a more varied range of oxidation (from one to four oxygen atoms). Second, the specialist herbivores excreted fewer metabolites of *p*-cymene compared to the generalists, which excreted numerous precursors of the extensively oxidised metabolites. Third, the role of secondary conjugation with glucuronic acid or glycine was of little importance in the specialist herbivores. We suggest that dietary limitations of specialist folivores favours the excretion of extensively oxidised metabolites of *p*-cymene, rather than glucuronide conjugates.

Degree of metabolite oxidation

Figure 8.1 and Table 8.1 show that the specialists excreted only extensively oxidised metabolites. This pattern is further clarified when metabolites are grouped according to the number of oxygen atoms acquired during oxidation, prior to any subsequent conjugation. Thus Cy 10, a glycine conjugated metabolite, was considered as having received two oxygens (as cumic acid). Figure 8.2 clearly shows that, with one exception, the specialists only excreted metabolites with three or more oxygen atoms added to the parent compound. The exception is the excretion of the glycine conjugate of cumic acid by the koala. Glycine conjugation prevented further oxidation of this two oxygen metabolite. Without this conjugation, there would probably have been no two oxygen metabolites of *p*-cymene excreted (since no free cumic acid was excreted). In contrast, the rat excreted a range of metabolites encompassing all degrees of oxidation, but predominantly the less oxidised metabolites. The metabolites excreted by brushtail possums were moderately oxidised (two to three oxygen atoms).

Therefore progression from generalist to specialist results in a distinct increase in the proportion of more extensively oxidised metabolites. The physical nature of monoterpenes is such that oxidations at different carbons is possible, allowing the formation of multiply oxidised metabolites. Given that monoterpenes such as *p*-cymene are always present in the eucalypt leaf diet of these folivores, this difference indicates an adaptation of detoxification mechanisms to diet.

Number of metabolites excreted

In the rat, we identified five major and four minor metabolites. As discussed in Chapter 2 - section 2.4, Walde *et al.* (1983) reported a total of 16 *p*-cymene metabolites in rat urine. The five major metabolites identified in our study corresponded to the five predominant metabolites reported by Walde, although there were differences in relative abundances.

In the brushtail possum, we identified a total of eight metabolites of which five were major and three were minor metabolites. In contrast, the specialist herbivores excreted fewer metabolites. In the ringtail possum only three major metabolites and a trace of a fourth (Cy 5) were detected and only three metabolites were found in the glider. Although six metabolites were detected in the koala, two metabolites accounted for the majority of the dose and another, Cy 11, was detected as a trace only.

The formation of fewer metabolites in the specialists suggests they have efficient oxidation pathways. In the generalists, excretion of less oxidised precursor metabolites in either their free or glucuronide form suggests that oxidation pathways are less efficient.

Conjugation

Glucuronidation. There was no evidence of glucuronidation of *p*-cymene metabolites in any of the specialists, yet significant amounts of metabolites were glucuronidated in the brushtail possum and rat. Glucuronidation is utilised by many animals due to its high capacity and its effectiveness in increasing the renal excretion of xenobiotics and endogenous waste compounds (Hirom *et al.* 1977; Caldwell 1982). It has been

reported that the glucuronidation pathway is particularly important in the brushtail possum as it has a low capacity to form sulphate conjugates, probably due to dietary deficiencies of sulfate (Roy 1963; Baudinette *et al.* 1980). This is also likely to be the case in the specialist folivores. Hinks and Bolliger (1956 and 1957) also noticed that eucalypt folivores presented high levels of glucuronic acid or glucuronides in their urine compared to other marsupials. Thus, it was reasonable to expect the hydrolysable conjugates identified in this study to be glucuronides. This was confirmed directly by measuring increases in urinary glucuronic acids in the rat and brushtail possum.

McLean *et al.* (1993) also found glucuronidation of terpenes, derived from dietary *E. radiata*, to be minimal in ringtail possums. When fed *E. radiata* for extended periods, the excretion of glucuronides increased over the first three days, but remained less than 30 % of the total amount of oxidised terpene metabolites. Chronic ingestion of *p*-cymene may therefore induce glucuronyl transferase enzymes in ringtails resulting in increased glucuronidation of monoterpenes, but the major proportion of metabolites is excreted unconjugated.

Glucuronidation was extensive, but variable, in the rat and brushtail possum (Figure 8.4). The rat excreted a greater portion of metabolites as conjugates after the lower dose than after the higher dose, whereas the trend was in the opposite direction for the brushtail possum. The dose dependent excretion in the rat suggests saturation of the glucuronidation pathway, which evidently has a higher capacity in the brushtail possum. It is possible that glucuronyl transferases are induced after exposure to monoterpenes such as *p*-cymene in brushtail possum, which may account for the increase in glucuronidation. However, possums were rested for at least two weeks before the second dose.

Historically, glucuronidation was considered to be the major detoxification pathway involved in the elimination of monoterpenes in the diet of eucalypt folivores (Hinks and Bolliger 1956 and 1957; Southwell 1974 and 1975). It is now clear that *p*-cymene undergoes minimal, if any, conjugation in the specialist eucalypt folivores. Conservation of the glucuronidation pathway in the metabolism and excretion of simple monoterpenes would preserve available glucuronidating capacity to assist the elimination of other PSMs which are not so readily or extensively oxidised (eg. small phenolic compounds). The large quantity of glucuronic acid excreted by the leaf-fed greater glider and koala supports the idea that glucuronidation is important in the elimination of other PSMs. This is further investigated in Part 2 of this thesis (Chapter 11 - section 11.3.2).

Glycine conjugation. The glycine conjugation pathway was active in all the species studied, as the glycine conjugate of benzoic acid, hippuric acid, was excreted in each case. Walde *et al.* (1983) reported the following glycine conjugates of *p*-cymene in rats: 2% of administered dose was excreted as cuminuric acid (Cy 10) and a trace as *p*-isopropenylbenzoylglycine (Cy 11). Trace amounts of Cy 10 were detected in the rat and trace amounts of Cy 10 and 11 in the brushtail possum. No glycine conjugated *p*-cymene metabolites were found in the ringtail or glider. Suprisingly, in the koala, approximately 7 % of the recovered metabolites was Cy 10. Southwell (1974) also identified Cy 10 in koala urine and suggested that this pathway was the major route of elimination. McLean *et al.* (1993) found no glycine conjugates of the carboxylic acid

metabolites of *E. radiata* terpenes in the ringtail possum. Interestingly, in the guinea pig, the major metabolite of *p*-cymene is the glycine conjugate, Cy 10 (Walde *et al.* 1983).

8.3.2. Urinary glucuronic acid excretion

Each animal species studied excreted some glucuronic acid. Direct comparison between species of total urinary glucuronic acid excretion, before and after dosing, was difficult as a number of factors were variable between experiments. It was necessary to minimise the resident time in metabolism cages for some animals, and for these there was no complete 24 h pre-dose urine sample was collected.

Diet was another major variable affecting overall glucuronic acid excretion. Species feeding on an artificial diet tended to excrete low levels of glucuronic acid while the glider and koala excreted very high levels, reflecting their eucalypt leaf diet (Table 8.4). Glucuronic acid excretion in the ringtail and glider demonstrate the effect of diet. Both species exhibited a similar excretion pattern of *p*-cymene metabolism with neither excreting significant amounts of *p*-cymene glucuronides. Yet, glucuronic acid levels were low in the ringtail, feeding on an artificial diet, whereas levels were very high in the glider feeding on *E. polycarpa.*

There was metabolic evidence of glucuronidation in the rat and brushtail possum. The percent of conjugated metabolites decreased in the rat after the higher *p*-cymene dose possibly suggesting dose dependent glucuronidation.

Correlations between molar glucuronic acid levels and molar metabolites conjugated were reasonable in each species. Reconciling conjugated metabolites and urinary glucuronic acid was only moderately successful for each species and for many individual animals was poor.

The absence of glucuronidation of *p*-cymene metabolites in the glider and koala is noteworthy considering the degree of glucuronuria. Koalas excreted about 2 g of glucuronic acid daily. Glucuronidation of other PSMs in the koala is considered in more detail in Part 2 of this thesis.

8.3.3. Ecological perspective of *p*-cymene metabolism

Metabolic strategies may be considered in the following way. For each species the ultimate aim of metabolism is to produce the most readily cleared metabolites. After initial oxidation, each species balances further oxidation with conjugation to produce a metabolite excretion pattern optimising metabolic costs. It seems that the brushtail possum can afford to conjugate less oxidised metabolites, via the glucuronidation pathway, rather than opting for further enzyme specific oxidation, preferred by the specialists.

We can only postulate on the reasons why specialists use a different strategy in the elimination of *p*-cymene compared to the generalist. Since renal excretion is enhanced by increased polarity, it is reasonable to assume that the greater the degree of oxidation, then the greater the rate of elimination of the metabolite. Increased polarity, whether achieved by radical oxidation or conjugation, presumably results in

the same overall enhanced capacity to excrete metabolites. It would therefore seem reasonable to assume that the different strategies employed by specialists, compared to generalist, must be based on some other factor, such as a balance of dietary energetics or enzyme availability.

The metabolic strategies used in the elimination of *p*-cymene may reflect the dietary classifications of each species studied. As mentioned previously, the marsupials studied cover the range of feeding niches from generalist to specialist. As the eucalypt component of the diet increases, the diet becomes nutritionally limited, as it contains low levels of nitrogen as well as being high in PSMs and fibre (Cork 1984; Foley 1987). Therefore, metabolic costs of metabolising such compounds could potentially become a significant factor in the metabolic pathways employed.

Herbivores require adaptations to successfully specialise on a potentially toxic and nutritionally poor diet such as eucalypt leaves. An enhanced capacity of liver and possibly other metabolically active tissues to extensively oxidise compounds such as *p*-cymene and other terpenes would support this hypothesis. The similar pattern and extent of oxidation of metabolites excreted by the ringtail possum and greater glider, which not only have a similar folivorous diet, but are also closely related as members of the Marsupialia family *Pseudocheiridae* (Flannery 1994), also adds weight to this hypothesis. Extensive oxidation of *p*-cymene enables the formation of highly polar metabolites which can be efficiently excreted without needing subsequent conjugation. This stratagem conserves the nutrients which would otherwise be required to form glucuronides and glycine conjugates.

The diet of the brushtail possum may be better nutritionally than that of the specialists due to the wider variety of food consumed (Freeland and Winter 1975). Greater variety would also result in the generalist herbivore being exposed to smaller amounts of a larger range of PSMs. A number of detoxification pathways with lower capacity would therefore be an advantage to the generalist herbivore, and our data showing a greater number of *p*-cymene metabolites in brushtail possums may support this. Freeland and Winter (1975) determined that brushtail possums were unable to maintain their body weight when fed a diet consisting solely of one species of *Eucalyptus*. They hypothesised that the cumulative dose of only a few PSMs exceeds the brushtail's detoxifying capacity and results in an inability to eat sufficient food to maintain body weight. Thus, it would seem the strategy employed by the brushtail possum requires a varied diet, in which the overall toxic load can potentially be great, but the detoxifying process is dispersed over numerous pathways. The results presented here show that a multiplicity of metabolic pathways also occurs with a single compound, *p*-cymene. At present, the enzymes involved in terpene metabolism are unknown although work is being conducted in this area (Pass *et al.* In press).

The rat would encounter low exposure to terpenoid compounds in its omnivorous diet. No refined mechanism for detoxifying these compounds would be expected to have developed. Significantly, the rat was the only species studied to excrete a mono-oxygenated metabolite of *p*-cymene, and this was its major metabolite.

8.3.4. Metabolite recovery

The recoveries of the *p*-cymene doses were relatively consistent for each species (Table 8.2), however, a significant amount of the administered dose remained unaccounted for. Possible explanations for the unaccounted portion of the dose are discussed below.

Only major metabolites were quantified and, therefore, the contribution of minor metabolites has not been taken into account. This is likely to be more significant in the case of the rats and brushtail possums which produced a number of minor metabolites. Cy 13 is an example of an unquantified metabolite occurring in each species. It was first identified in the koala urine, which was the final experiment in this series. Methylated Cy 13 was too polar to chromatograph successfully by GC/MS. It was only detected in the koala urine because of its abundance, which resulted in a poorly formed peak. Further derivatisation of one of the alcohol groups improved its chromatography. It was then specifically searched for, and found, retrospectively in urine extracts from each of the other species. No attempt was made to quantify this metabolite in the other species, yet comparisons to peak sizes of other metabolites suggest it may have accounted for a few percent of the recovered dose in some animals. There is potential for other undetected metabolites, too polar to be extracted and chromatographed with the conditions used. However, this explanation is unlikely as all metabolic oxidations are likely to result in hydroxy and carboxylic acid metabolites, both of which are detectable by the derivatisations described.

The possibility of unchanged *p*-cymene being excreted in urine, faeces or expired air should be considered. As expected, unchanged *p*-cymene was not detected in the urine of any species. Traces were found in faeces of brushtail possums after oral administration by Southwell *et al.* (1980), but were not detected in this study. On the other hand, because of the volatile nature of *p*-cymene it could be expected to be detected in expired air. Lee (1987) reported that a small fraction of *p*-cymene absorbed into the blood of humans was excreted unchanged in expired air.

Urinary excretion was not followed after the second day, so any slowly-excreted components of the dose would also have been missed.

Glucuronic acid conjugates, under appropriate urinary conditions, can undergo intramolecular rearrangement with the resultant molecular structure being resistant to hydrolysis by β-glucuronidase (Dickinson *et al.* 1984; Spahn *et al.* 1989). Alkaline urine provides favourable conditions for this rearrangement. Rearranged glucuronides would therefore not be extracted and would remain unmeasured. However, storage conditions (acidic pH and low temperature) would be expected to minimise the potential for this to have occurred in those species in which glucuronidation was found.

Biliary excretion of conjugated metabolites was investigated by analysing faecal samples. However, no faecal metabolites were identified in any species. The molecular weight of *p*-cymene and its glucuronides are 134 and 243 - 273, respectively. Although the molecular weights of *p*-cymene metabolite glucuronides are borderline for biliary excretion (Pratt and Taylor 1990), the glucuronide of at

least one terpene, *l*-menthol, is excreted by this route in the rat (Yamaguchi *et al.* 1994).

8.3.5. Terpene metabolism

The metabolic pathway suggested for *p*-cymene in Chapter 2 - Figure 2.1 is based on the assumption that some metabolites are precursors of the more highly oxidised metabolites. These minor, or pre-cursor, metabolites provide an insight into the metabolic pathways which result in the final, extensively oxidised, metabolites. The absence of precursors in the greater glider, ringtail possum and koala suggests there is no leakage of the intermediate compounds and therefore they are not available for renal excretion.

As discussed in Chapter 1 - section 1.5.3.1, carboxylic acid metabolites are likely to be products of two enzyme systems. Firstly, microsomal CYP enzymes are responsible for the formation of alcohol metabolites (G. Pass, personal communications). The 7, 8 and 9 carbon positions of *p*-cymene are all reactive sites for CYP oxidation. Secondly, cytosolic alcohol and aldehyde dehydrogenases are likely to be responsible for the formation of carboxylic acids from the products of CYP. The large number of precursor metabolites possibly suggest that the oxidation reactions proceed via a series of single step reactions. As the efficiency of the enzyme system increases, particularly the cytosolic dehydrogenases, fewer of the precursor alcohol metabolites would be expected.

Others have examined the stereochemistry of terpene metabolism. Matsumoto *et al.* (1992) studied the metabolism of *p*-cymene in rabbits and found that the enzymatic oxidation of *p*-cymene occurred stereoselectively. Stereoselective metabolism has also been found in the metabolism of 1,8-cineole in brushtail possums (Carman and Klika 1991; Carman and Klika 1992; Carman *et al.* 1994; Carman and Rayner 1994). It would, therefore, be reasonable to expect that some of the metabolites excreted in the animals studied here would also show chiral characteristics, but this requires further investigation.

Current investigations into marsupial CYP enzymes may eventually provide an insight into specificities and adaptations of these enzymes in the metabolism of terpenes, particularly in eucalypt folivores. A review of *in vitro* studies on marsupials CYPs was presented in Chapter 1 - section 1.5.3. The role of alcohol and aldehyde dehydrogenases, however, still remains to be studied, but the species specific pattern of metabolite excretion presented here suggest that specific adaptations of this enzyme system may prove to be equally important in the metabolism of terpenes in these species.

8.3.6. Elimination rate

It was hoped that information on the excretion rate of *p*-cymene in each species would be obtained from these experiments by measuring the percent of the dose recovered in the first 24 h. However, 24 h urine collections were too long to provide information on the excretion rate of *p*-cymene, which is rapidly metabolised (Table 8.3). Instead, urine output directly affected the 24 h post-dose excretion of *p*-

cymene metabolites. Urine output was, in turn, dependent on fluid intake and in some cases this was highly variable, as in the case of the lower dose of *p*-cymene in the brushtail possum where some animals did not urinate in the first 24 h after the dose.

8.3.7. Variability of results

Consideration of the possible sources of variability in these results suggests that most was due to normal inter-animal variation. Experimental variability was minimal as established by the reproducibility of calibration curves (see Chapter 2 - section 2.2.6.1). Calibration samples and real samples underwent the same analytical procedures. Although every attempt was made to be consistent with dosing and sample collection, it is the nature of animal experimentation, where co-operation is not guaranteed, that significant variability will result.

8.3.8. Limitations

Conclusions made in a comparative study such as this are necessarily limited. An assumption is made concerning the equality of the "toxic challenge" administered to each species. A dose of 1.49 mmol/kg of *p*-cymene may be significantly more challenging for the rat than for the koala. Furthermore, the results observed from a single dose of one terpene may not accurately reflect what occurs in chronic dosing of mixed terpenes, as in a natural diet. Therefore, conclusions made are confined by the choice of the dosage regimen. Experience with chronic and/or larger dosing regimens are required to make more definite conclusions and are further investigated in Part 2 of this thesis. Nonetheless this study has provided the essential tools necessary to examine constraints on detoxification in mammals.

PART 2

COMPARATIVE METABOLISM OF 1,8-CINEOLE DURING CHRONIC INGESTION IN THE BRUSHTAIL POSSUM AND KOALA

CHAPTER 9

METHOD DEVELOPMENT OF 1,8-CINEOLE METABOLITE ASSAY

9.1. Introduction

The findings reported in Part 1 of this thesis were concerned with the metabolic fate of a single dose of the monoterpene, *p*-cymene, in a comparative study of five animal species. This provided basic information on the metabolic fate of *p*-cymene. However, single bolus doses do not reflect the pattern of chronic ingestion in a natural diet. Part 2 of this thesis is concerned with firstly identifying the metabolic fate of 1,8-cineole in the brushtail possum and the koala (Chapter 9) and then looking more closely at the pattern of metabolism during prolonged feeding on a diet containing 1,8-cineole. In Chapter 10 three feeding trials were conducted in which brushtail possums were challenged with 1,8-cineole containing diets. Detoxification strategies were investigated by analysing the pattern of excretion of urinary metabolites. The findings were then compared with observational data on 1,8-cineole ingestion. Chronic 1,8-cineole ingestion in koalas was opportunistic. *p*-Cymene dosing experiments in Part 1 required the koalas to feed on a *Eucalyptus* species which contained minimal *p*-cymene. *E. cephalocarpa* was selected and, although the terpene content was low, the terpene profile consisted of > 90 % 1,8-cineole. This allowed us to examine 1,8-cineole metabolism in the same urine samples as those collected for the *p*-cymene dosing experiments and the results are reported in Chapter 11.

1,8-Cineole is the most abundant terpene found in *Eucalyptus* spp. In many species it is the dominant monoterpene, often accounting for more than 90 % of the terpene content (Guenther 1950; Boland and Brophy 1991; Li *et al.* 1994). Inevitably, marsupial eucalypt folivores encounter 1,8-cineole in their diet, often consuming many grams per day. These significant quantities would be toxic to many animal species, including humans (Jenner *et al.* 1964; Patel and Wiggins 1980; Webb and Pitt 1993; Barnes 1996). Therefore the marsupial eucalypt folivores have necessarily developed detoxification strategies to cope with the challenge. For this reason it is an ideal terpene for studying in detail the detoxification strategies employed by the brushtail possum, a generalist herbivore and the koala, a specialist eucalypt folivore.

1,8-Cineole produced a very complex array of metabolites in the brushtail possum and koala. Many of the metabolites have not previously been reported in the literature.

Development of assay methods used to extract and quantify metabolites of 1,8-cineole in both urine and faeces, as well as direct measurement of urinary glucuronic acid, are described in this chapter.

9.2. Methods

9.2.1. Extraction, derivatisation and analysis of urine samples

The method used for extraction of both brushtail possum and koala metabolites from urine was a refinement of that described in Part 1 for analysis of *p*-cymene metabolites.

Urine samples were analysed to quantify free and total metabolites excreted by each species. Free metabolites were analysed by accurately measuring thawed urine samples into tapered centrifuge tubes. An appropriate dilution of urine with distilled water was made for each experiment. Urine dilutions are tabulated in Table 9.1.

Table 9.1. Urine dilutions required for the analysis of 1,8-cineole metabolites in each 1,8-cineole feeding experiment in Chapter 10 and 11.

	Volume (µl)			Dilution
	Urine	Water	Total	factor
Brushtail possums				
Experiment 1	250	250	500	2
Experiment 2	100	400	500	5
Treatment	100	400	500	5
Washout	200	300	500	1.5
Experiment 3	100	400	500	5
Koalas				
Leaf diet	100	400	500	5

To each diluted sample, internal standard was added (0.5 mg 2,5-dimethylbenzoic acid in 50 µl methanol). The urine was acidified to pH 1 with 20 µl 5 M HCl and then extracted with three sequential volumes of ethyl acetate (1 x 1 ml and 2 x 0.5 ml). Vortexing for 30 s and subsequent centrifuging at 2000 rpm for 5 min ensured thorough mixing and separation of ethyl acetate and urine. Carboxylic acid metabolites were then derivatised to their methyl esters. Approximately 100 µl of the ethyl acetate extract was transferred to a screw capped 5 ml glass vial and 200 µl diazomethane added (Chapter 2 - section 2.2.1). The vial was capped and allowed to sit for 30 min in an ice bath. Excess diazomethane was evaporated under a stream of N_2 at 40°C and the final volume readjusted to approximately 100 µl with ethyl acetate.

Because of the polarity of many metabolites, a second derivatisation with BSTFA to form the TMS derivatives of alcohol and hydroxyl groups was necessary for the resolution of these metabolites by gas chromatography. Aliquots (20 µl) of the methylated ethyl acetate extract were measured into 100 µl glass inserts in septum sealed crimp cap autosampler vials and 20 µl BSTFA added. Vials were sealed and heated at 70°C for 20 min before GC analysis.

Total metabolites were measured by hydrolysing the urine before extracting. The same urine dilutions and internal standard were used as for the free metabolite assays. To diluted urines 100 µl of 1.1 M acetate buffer and 25 µl extract of *Helix pomatia* (β-glucuronidase plus aryl sulphatase) was added. Samples were heated at 37.5°C overnight in a waterbath.

After incubation, samples were treated identically to free metabolite samples except that 40 µl of 5 M HCl was required to acidify the acetate buffer. Also, the formation of emulsions in the ethyl acetate phase after vortexing required physical disruption by stirring with a glass pipette and re-centrifuging to dissipate the emulsion.

9.2.2. Gas chromatography and mass spectrometry

Gas chromatography of urine and faecal extracts was carried out on the GC/MS and capillary column described in Chapter 2 - section 2.2.2. GC/MS operating conditions were: split injector (1:10) at 250°C, injection volume 2 µl, He carrier gas (12 psi), oven 50°C for 1 min then 10°/min to 290°C and held for 2 min, detector 290°C, solvent delay of 2 min for methylated only extracts and 6 min for methylated plus TMS derivatised extracts, EM voltage 600 and selected mass range 40 - 600 amu.

The Chemstation software was modified to enable exporting of mass spectra to the NIST MS Search Program (details in Chapter 2 - section 2.2.2), enabling searching of both the NIST MS library and the custom terpene metabolite library of mass spectra.

9.2.3. Identification of metabolites

Mass spectral data for a number of 1,8-cineole metabolites have been published in the literature. The majority of published data are from a chemistry research group at the University of Queensland, headed by Dr Ray Carman. Metabolite data published by this group (as well as other authors) are summarised in Table 9.2. and were entered into a custom terpene metabolite mass spectra library. Mass spectra derived from 1,8-cineole metabolites could then be compared, using probability based comparisons, to these mass spectra. For the purpose of this study 1,8-cineole metabolites were assigned short names (Ci 1 to 22; see Table 9.3 and Figure 9.6 for metabolite names and structures).

Gas chromatography mass spectrometry. GC/MS of methylated-only urine extracts from brushtail possums fed 1,8-cineole resulted in chromatograms with many poorly resolved 1,8-cineole derived peaks (Figure 9.1-A). Before any progress could be made on identifying these compounds, their chromatography had to be improved. Development of the double derivatisation method which produces both methyl esters and TMS derivatives resolved most of the chromatography difficulties.

Table 9.2. Published mass spectral data for 1,8-cineole metabolites reported in possums. Also, where specified, a selection of metabolites from other organisms and some synthesised 1,8-cineole compounds.

Metabolite	Reference	MW	Significant EI ions (*m/z*) *Relative abundance (%)*																					
9-hydroxycineole	Carman & Klika, 1992	170	140	139	121	96	95	81	79	71	69	67	57	55	53	45	43	41	39					
			5	*47*	*3*	*2*	*20*	*8*	*2*	*15*	*1*	*7*	*2*	*7*	*4*	*4*	*100*	*10*	*8*					
9-hydroxycineole	Flynn & Southwell, 1979	170	170	140	139	95	43																	
			0.2	*5*	*51*	*24*	*100*																	
9-cineolic acid	Carman & Klika, 1992	184	140	139	95	93	81	71	67	55	53	45	43	41	39									
			5	*53*	*27*	*5*	*5*	*15*	*8*	*8*	*6*	*7*	*100*	*11*	*11*									
9-cineolic acid (methyl ester)	Flynn & Southwell, 1979	198	198	183	140	139	95	81	71	43														
			0.4	*2.6*	*9*	*84*	*29*	*5*	*14*	*100*														
7-hydroxycineole	Bull *et al.*, 1993	170	170	155	139	137	112	111	94	93	84	83	81	79	69	67	59	55	53	43	41			
			7	*54*	*15*	*18*	*15*	*100*	*26*	*68*	*30*	*41*	*25*	*38*	*77*	*25*	*36*	*36*	*18*	*84*	*54*			
7-cineolic acid	Bull *et al.*, 1993	184	169	151	126	123	111	84	83	82	81	80	79	77	69	67	59	55	45	43	41	39		
			66	*9*	*14*	*23*	*33*	*25*	*23*	*18*	*35*	*32*	*28*	*19*	*33*	*22*	*22*	*33*	*69*	*100*	*91*	*38*		
7-cineolic acid (methyl ester)	Bull *et al.*, 1993	198	198	197	154	151	139	126	123	111	109	108	84	83	81	80	79	69	67	55	53	45	43	41	39
			12	*100*	*26*	*50*	*15*	*16*	*41*	*36*	*18*	*16*	*15*	*39*	*43*	*27*	*28*	*68*	*16*	*46*	*18*	*79*	*91*	*5*	*26*
2α,4-dihydroxy-1,8-cineole	Carman & Rayner, 1994	186	186	143	142	124	110	109	99	85	84	71	70	69	67	59	58	55	43	42	41	39		
			5	*6*	*58*	*11*	*9*	*25*	*34*	*11*	*22*	*60*	*13*	*16*	*10*	*16*	*26*	*15*	*100*	*11*	*30*	*14*		
2,9-dihydroxy-1,8-cineole	Carman *et al.*, 1994	186	156	155	137	124	109	95	93	84	82	81	71	67	55	43	41							
			3	*36*	*5*	*7*	*11*	*11*	*18*	*13*	*9*	*9*	*12*	*9*	*9*	*100*	*14*							
2,10-dihydroxy-1,8-cineole	Carman *et al.*, 1994	186	156	155	137	124	109	95	93	84	82	81	71	67	55	43	41							
			3	*34*	*4*	*10*	*13*	*10*	*16*	*14*	*10*	*8*	*12*	*9*	*9*	*100*	*14*							
7,9-dyhydroxy-1,8-cineole	Carman & Garner, 1996	184	156	155	137	109	95	94	93	79	67	45	43	41										
			2	*17*	*13*	*6*	*7*	*5*	*50*	*12*	*10*	*14*	*100*	*16*										
2α,7-dihydroxy-1,8-cineole	Carman & Garner, 1996	184	169	168	143	142	140	124	124	112	111	109	93	92	87	83	69	67	55	43	41			
			1	*9*	*3*	*27*	*4*	*5*	*8*	*8*	*100*	*10*	*20*	*22*	*12*	*36*	*93*	*22*	*38*	*51*	*62*			
2β,4-dihydroxy-1,8-cineole synthesised only	Carman & Rayner, 1994	186	186	153	142	124	110	109	99	95	85	84	83	81	81	70	69	59	58	55	43	41	39	
			4	*3*	*72*	*13*	*13*	*32*	*37*	*10*	*12*	*26*	*11*	*10*	*51*	*12*	*16*	*17*	*36*	*14*	*100*	*30*	*10*	
4-hydroxy-2-oxo-1,8-cineole synthesised only	Carman & Rayner, 1994	184	184	157	156	141	123	99	98	85	84	83	71	69	59	58	57	55	43	41	39			
			1	*3*	*27*	*28*	*5*	*30*	*48*	*22*	*13*	*71*	*21*	*22*	*12*	*12*	*16*	*13*	*100*	*37*	*19*			
2β-hydroxy-1,8-cineole pyrgo bark beetle	Southwell *et al.*, 1995	170	170	137	126	111	108	93	83	71	69	43												
			5	*2*	*50*	*38*	*75*	*40*	*25*	*75*	*45*	*100*												
2-hydroxy-1,8-cineole *Pseudomonas flava*	MacRae *et al.*, 1979	170	170	126	111	108	93	83	71															
			47	*100*	*64*	*96*	*45*	*47*	*96*															

MW = molecular weight.

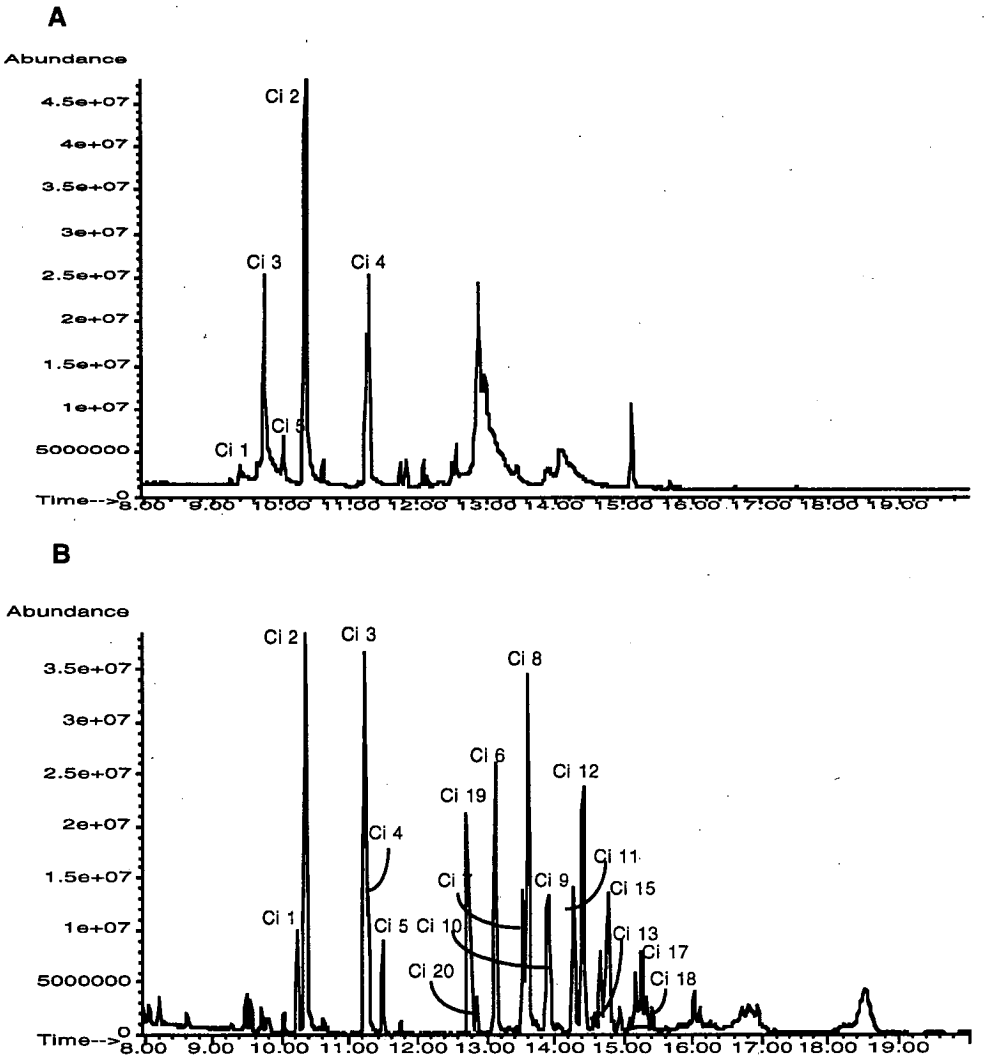


Figure 9.1. TIC chromatograms from GC/MS of brushtail possum urine extracts comparing derivatisation methods. **A)** Chromatography of extract after derivatisation of carboxylic acids to their methyl esters results in poor resolution of many metabolites. **B)** Double derivatisation of carboxylic acid and hydroxy moieties of metabolites to their methyl ester and TMS forms, respectively, improves their chromatography markedly. GC conditions are described in section 9.2.2. Metabolites are labelled with assigned names. See Table 9.3 for identification of individual metabolites.

Reference standards for a number of metabolites were kindly donated by Dr Ray Carman. Metabolites donated had been either synthesised or isolated from brushtail possum urine and were: 9-hydroxycineole, 9-cineolic acid, 2,4-dihydroxycineole, 2-hydroxycineole and 7-cineolic acid. These standards were especially valuable as they could be derivatised in the same manner as the urine extracts and therefore provided reference mass spectra for the derivatised metabolites. These mass spectra were added to the custom terpene metabolite library of mass spectra.

Mass spectral data were to be the main method of determining 1,8-cineole metabolites in this study. However, the metabolism of 1,8-cineole in the brushtail

possum and koala revealed a large array of metabolites. Many major and very polar metabolites were novel. Investigation using several modes of mass spectrometry determined that all of the unknown metabolites were isomers of dihydroxycineole and hydroxy cineolic acids. Plus, dicineolic acid was identified in the koala urine. Allocating metabolites to groups according to the extent of their oxidation satisfied the requirements of this research.

The derivative of each metabolite had to be carefully considered when mass spectra were used for identification since metabolites could be susceptible to either one or both derivatisations. For example, the hydroxy cineolic acids generally formed both methyl ester and TMS derivatives. However, two metabolites in this group did not accept the TMS group due to steric hindrance. For simplicity, the electron ionisation (EI) GC/MS mass spectra are only reported for metabolites in their fully derivatised state. Most metabolites did not produce molecular ions (M^+) after EI-MS, therefore chemical ionisation (CI) GC/MS was used to determine protonated molecular ions ($[M+H]^+$) from which empirical formulae were derived.

CI-GC/MS and high resolution MS analyses were carried out on the instrument described in Chapter 7 - Section 7.2.5. GC and MS conditions were described Section 9.2.2.

Reference mass spectra and GC retention times were obtained for all metabolites. Where possible, these characteristics were obtained from isolated metabolites. Otherwise, they were obtained directly from metabolites in urine extracts. Once metabolites had been identified and their mass spectrometry characterised, all metabolites could be separated in the chromatogram by extracting mass chromatograms of diagnostic ions (Table 9.3). This also enhanced the detection of minor metabolites which were otherwise difficult to detect from the background noise of the total ion current (TIC) chromatogram. It was therefore necessary to relate the abundance of the diagnostic ions to the actual amount of metabolite to allow quantitation of metabolites from calibration curves prepared for structurally similar metabolites. The abundance of the diagnostic ion as a fraction of the TIC was calculated from a good quality TIC chromatogram produced for each metabolite. The inverse of each fraction could then be multiplied to future diagnostic ion abundances to calculate the TIC (Table 9.3).

Liquid chromatography mass spectrometry (LC/MS). LC/MS of possum and koala urine confirmed molecular weights for each group of metabolites and their respective glucuronides. Mass spectrometry - mass spectrometry (MS/MS) of 1,8-cineole derived metabolites provided further mass spectral evidence. Urine samples were filtered (0.45 micron) after dilution (1:10). The LC/MS used was a Waters Alliance 2690 HPLC coupled to a Finnigan LCQ using atmospheric pressure chemical ionisation (APCI) and electrospray ionisation (ESI). The chromatography was carried out on a Waters NovaPak C18 analytical column. Mobile phase was 10 % methanol/90 % water (with 2 % acetic acid) and graded to methanol 100 % at 40 min, flow rate of 0.8 ml/min and injection volume 15 µl. Positive ion APCI was used to identify most 1,8-cineole metabolites in both the brushtail possums and koala.

Table 9.3. Assigned metabolite names and derivatisations for each 1,8-cineole metabolite found. Diagnostic ions (DI) used to extract mass chromatograms and the conversion factor used to calculate the TIC from the diagnostic ion abundance are also shown.

Assigned name	Metabolite	Derivative[1]	Species[2]	Diagnostic ion (DI)	DI - TIC[3] factor
IS	2,5-dimethylbenzoic acid	M	-	164	9.05
Ci 1	hydroxycineole	TMS	BP	159	10.47
Ci 2	9 cineolic acid	M	BP/K	139	2.83
Ci 3	9-hydroxycineole	TMS	BP/K	139	2.97
Ci 4	7 cineolic acid	M	BP/K	183	7.08
Ci 5	7-hydroxycineole	TMS	BP/K	183	14.91
Ci 6	hydroxy acid cineole A	M+TMS	BP	227	13.36
Ci 7	hydroxy acid cineole B	M+TMS	BP	227	12.31
Ci 8	hydroxy acid cineole C	M+TMS	BP	227	9.81
Ci 9	hydroxy acid cineole D	M+TMS	BP	227	13.46
Ci 10	dihydroxy cineole A	TMS	BP	243	11.10
Ci 11	dihydroxy cineole B	TMS	BP	227	5.01
Ci 12	hydroxy acid cineole E	M+TMS	BP	227	12.36
Ci 13	hydroxy acid cineole F	M+TMS	BP/K	227	13.91
Ci 13	7-hydroxy-9-cineolic acid	M	BP/K	155	10.58
Ci 14	dihydroxycineole C	TMS	BP	227	6.90
Ci 15	hydroxy acid cineole I	M+TMS	BP	227	22.89
Ci 17	9-hydroxy-7-cineolic acid	M+TMS	BP/K	227	3.50
Ci 18	hydroxy acid cineole H	M+TMS	BP	227	12.01
Ci 19[3]	hydroxy acid cineole J	M	BP	155	7.00
Ci 20[3]	hydroxy acid cineole K	M	BP	155	11.22
Ci 22	7,9-dicineolic acid	M	K	183	5.07

[1]Methyl ester (M) or TMS ether (TMS).

[2]Species in which metabolites were found: BP = brushtail possum and K = koala.

[3]The conversion of diagnostic ion abundances to TIC abundances is described in the text - Section 9.2.3.

[4]Ci 19 and 20 did not form the TMS derivative due to steric hinderance at the OH group.

IS = internal standard

Negative ion ESI was used to investigate unknown glucuronides in the koala urine. Both hydrolysed and unhydrolysed urine samples were analysed. The same HPLC conditions were used as for positive ion APCI mode described above.

Urine was hydrolysed with extract of *Helix pomatia* as described for total metabolite analyses. It was then diluted (1:10) for LC/MS analysis.

Nuclear magnetic resonance (NMR). NMR was used to elucidated the structure of Ci 13, a hydroxy cineolic acid isolated from koala urine. Both ^{1}H-NMR and ^{13}C-NMR were performed and interpreted by Dr Evan Peacock, Central Science Laboratory, University of Tasmania. They were recorded on a Varian 400 MHz Widebore Inova system on a 4 nucleus 5 mm solution probe and z-axis inverse pulsed field gradient. The isomeric structure and relative stereochemistry were determined on the basis of short and long range gradient-COSY, gradient-HMQC/HMBC, DEPT and gradient-NOESY experiments.

9.2.4. Isolation of metabolites

Quantitation of metabolites required reference standards for metabolites. The complexity of metabolites excreted by possums and koalas meant it was not practical to have standards for individual metabolites. Metabolites were isolated where possible, otherwise quantitation relied on calibration curves prepared for a structurally similar metabolite.

Two metabolite mixtures were isolated from brushtail possum urine. The first was a mixture of 9- and 7-cineolic acids, and the other a mixture of 9- and 7-hydroxycineole. Further separation of these two mixtures was unsuccessful. The proportion of each metabolite was measured by GC/MS allowing the preparation of separate calibration curves.

One hydroxy cineolic acid metabolite, Ci 13, which dominated the metabolite profile of koala urine was isolated. The calibration curve prepared from this metabolite was used to quantify all other hydroxy cineolic acids.

Detection of 1,8-cineole metabolites by UV-dependent methods was not possible since these metabolites did not absorb UV strongly (UV absorbance maxima were about 194 nm for hydroxycineole metabolites), nor were they susceptible to other trialed indicators (vanillin spray - 1 % vanillin in conc sulphuric acid, and iodine) that would allow their detection on TLC. This made separation by HPLC and TLC difficult.

9.2.4.1 Isolation of metabolites from brushtail possum urine

Brushtail possum urine, from feeding trials containing high concentrations of 1,8-cineole (Chapter 10), was combined for the purpose of isolating metabolites. Approximately 1000 ml of urine was thawed and hydrolysed by adding 100 ml of 1.1 M acetate buffer, 3 ml of extract of *Helix pomatia* and 100 mg of sodium fluoride (antibacterial) in a large conical flask and heating at 37.5°C for 5 days.

Sufficient hydrolysis had occurred after 5 days, determined by substantial increases in the amounts of Ci 3 (9-hydroxycineole) and Ci 7 (7-hydroxycineole) when a small volume of the urine was extracted and analysed by GC.

The urine was then fractionated into strong acid, weak acid and neutral fractions. Approximately 500 ml of hydrolysed urine was placed in a separating funnel and acidified to pH 1 with 100 ml of 5M HCl. Urine was extracted with ethyl acetate (3 x 150 ml). The resultant extract was washed sequentially with chilled 5% aqueous $NaHCO_3$ (4 x 50 ml) to re-extract strong acids and 5% aqueous NaOH (4 x 50 ml) solutions to re-extract weak acids, leaving a neutral extract. The basic solutions were acidified with 20 and 25 ml of concentrated HCl respectively. The acidified aqueous fractions were then back extracted into ethyl acetate using 3 x 100 ml washes for each fraction.

A small portion of each fraction was methylated for analysis by GC/MS. The strong acid fraction contained predominantly 9-cineolic acid and 7-cineolic acid plus several other minor peaks.

Surprisingly, only very small amounts of the hydroxy cineolic acids were present in the ethyl acetate extract of the strong acid fraction. Nor were they accounted for in either the weak acid or neutral fractions. They were finally located in the acidified $NaHCO_3$ (ie. the remaining aqueous strong acid fraction). They had not back extracted into ethyl acetate with the monoacids, presumably due to the influence of salts in the solution and their polarity. They were successfully extracted using a more polar solvent (dichloromethane and *n*-propanol; 50:50). The dichloromethane/*n*-propanol solvent was removed by evaporation and the residue weighed (358 mg). The mixture was complex and contained all the hydroxy cineolic acids. It was considered too difficult to separate individual hydroxy cineolic acids from this mixture. It was decided to isolate Ci 13 (7-hydroxy-9-cineolic acid, see Section 9.3.1 for identification of this metabolite and Figure 9.10 for its chemical structure), as it dominated koala urine, and then to use the isolate as a standard for quantifying all other hydroxy cineolic acids.

The neutral fraction contained predominantly 9-hydroxycineole and small amounts of 7-hydroxycineole. Another unfamiliar, but major, peak was also present with a similar mass spectrum to that of 9-hydroxycineole. The weak acid fraction contained no peaks at all and was discarded. Separation and purification of each fraction is described below.

Strong acid fraction. The first step in separating and purifying 9- and 7-cineolic acids used TLC. Preliminary tests with small analytical TLC plates determined that the best separation of UV fluorescence quenching and vanillin sensitive compounds (ie. non 1,8-cineole derived compounds such as hippuric acid and phenyl acetyl glycine) was achieved using unmethylated extracts in a mobile phase of ethyl acetate/hexane 50:50. It was assumed that this would result in the best separation of the non-visible cineolic acids.

pTLC plates were prepared as in Chapter 2 - section 2.2.4. Concentrated strong acid fraction was applied to two pTLC plates (70 cm wide). Six zones (0 - 5) were removed according to bands detected by UV fluorescence quenching and vanillin spray (Figure 9.2-A). Each zone was redissolved in sequential methanol washes (3 x 15 ml). Methanol extracts were evaporated to dryness and the residue redissolved in ethyl acetate, methylated and analysed by GC. Both 9- and 7-cineolic acid were present predominantly in zone 4 (Figure 9.2-A).

Further attempts by TLC to separate 9- and 7-cineolic acid as either their acid or methyl ester forms, using various solvents, was unsuccessful and a mixture of the two metabolites was used for quantitation. The purity of the combined acids in the final standard used for quantitation was 98.4 % and the proportions of 9-cineolic acid to 7-cineolic acid were 91.7 and 8.3 % respectively.

Neutral fraction. TLC was also used for separating and purifying Ci 3 and 5 (9- and 7-hydroxycineole). The same TLC system as used for the separation of the acids was used for the hyroxycineoles (Figure 9.2-B).

Eleven zones (0 - 10) were removed according to bands detected by UV quenching and vanillin spray. These were redissolved in sequential methanol washes (3 x 15 ml). Methanol extracts were evaporated to dryness and the residue redissolved in

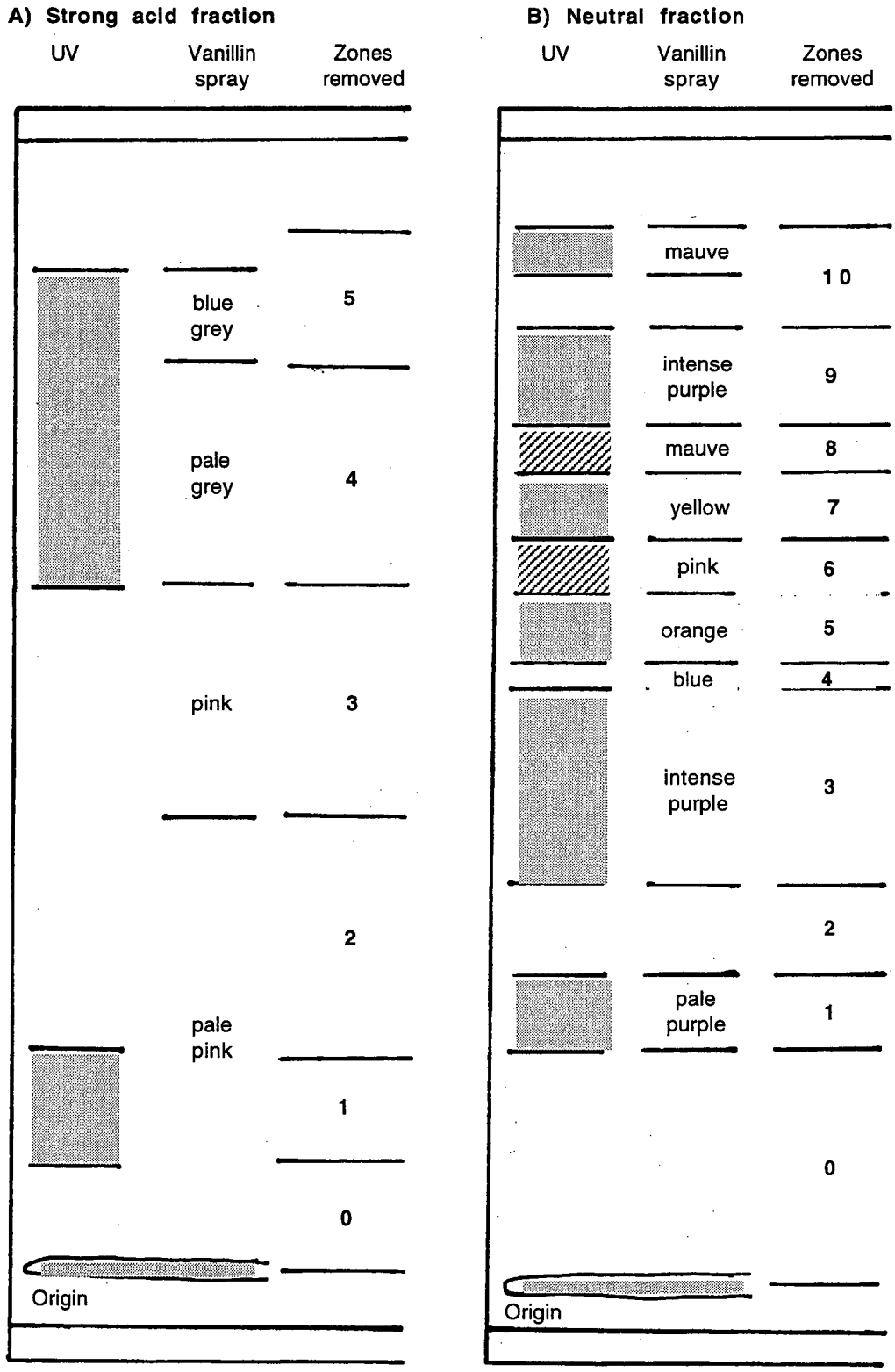


Figure 9.2. Schematic representation of developed pTLC plates for **A)** strong acid fraction (7- and 9-cineolic acid were located in Zone 4) and **B)** neutral fraction (7- and 9-hydroxycineole were located in Zones 3, 4 and 5) of brushtail possum urine. The zones removed are indicated on the right hand side of each plate. pTLC conditions are described in section 9.2.4.1.

ethyl acetate and analysed by GC/MS. Both 9- and 7-hydroxycineole were present in zones 3, 4 and 5. Zone 3 was the purest (97.4 %) and the relative proportions of 9-hydroxycineole and 7-hydroxycineole were 85.6 and 14.4 % respectively.

The unknown compound was found in zone 9 (Figure 9.2-B). It was dissolved in ethyl acetate and its mass spectrum analysed. Mass spectral data was *m/z* (%): 213.1470 ($C_{12}H_{21}O_3$, calculated mass 213.296, M^+ (tr)), 139 (52), 109 (4), 95 (15), 43 (100). The structure was proposed to be 9-hydroxy acetate cineole, an artefact of 9-hydroxycineole.

9.2.4.2 Isolation of metabolite from koala urine

Urine (200 ml) from koalas fed *E. cephalocarpa* (see Chapter 11) was fractionated into strong acid, weak acid and neutral fractions in a similar manner as the brushtail possum urine above. A sample of each fraction was methylated and analysed by GC/MS (conditions described in Section 9.2.1). The weak acid and neutral fractions contained no peaks at all and were discarded. The strong acid fraction contained multiple peaks including the dominant hydroxy cineolic acid metabolite, Ci 13. It was decided to concentrate on isolating Ci 13 to allow its quantitation in koala urine as well as being a standard for all hydroxy cineolic acids in brushtail possum urine.

The next step in isolating Ci 13 used pTLC. Glass plates were prepared as in Chapter 2, section 2.2.4. Preliminary tests with analytical TLC plates determined that the best separation of fluorescence quenching compounds in the extract was achieved in a mobile phase of 100 % ethyl acetate and it was assumed that this would also result in the best separation of the non-visible 1,8-cineole metabolites.

Concentrated strong acid fraction was methylated with excess diazomethane and applied to 75 cm wide pTLC plates and developed. The plates were visualised by UV, vanillin spray and visible light (Figure 9.3). Ten zones were removed according to bands detected by one or more of the detection methods and redissolved in sequential methanol washes (2 x 15 ml and 1 x 10 ml). Methanol extracts were evaporated to dryness and the residue redissolved in ethyl acetate and analysed by GC. Ci 13 was present in zones 5 and 6 and these were combined. Ci 13 yield was about 400 mg and purity, measured by GC/MS, was 85%.

Final purification was by pHPLC. Initial chromatography was established on an analytical HPLC column (instrument and conditions were described in Chapter 2, section 2.2.4). The mobile phase was water/methanol 55:45, flow rate 1 ml/min and detector wavelength 210 nm. Although Ci 13 was not expected to produce a strong peak, good separation of UV absorbing compounds was indicated by the separation of approximately 20 peaks.

The method was transferred to a pHPLC system. The HPLC used was a Waters M-45 Solvent Delivery System coupled to a Waters Series 440 Absorbance Detector (214 nm) (Waters Associates, Inc, Milford, Massachusetts, USA) and LDC/Milton Roy CI-10B Integrator and was fitted with a Waters PrepPak Cartridge 25 x 100 mm, prep NovaPak HR C18 6 µm 60 Å plus a GuardPak Cartridge as a guard column. The column was radially compressed in a Waters PrepLC 25 mm Module at < 1500 psi. The HPLC pump was operated at a flow rate of 9 ml/min with the same mobile phase as used for the analytical system.

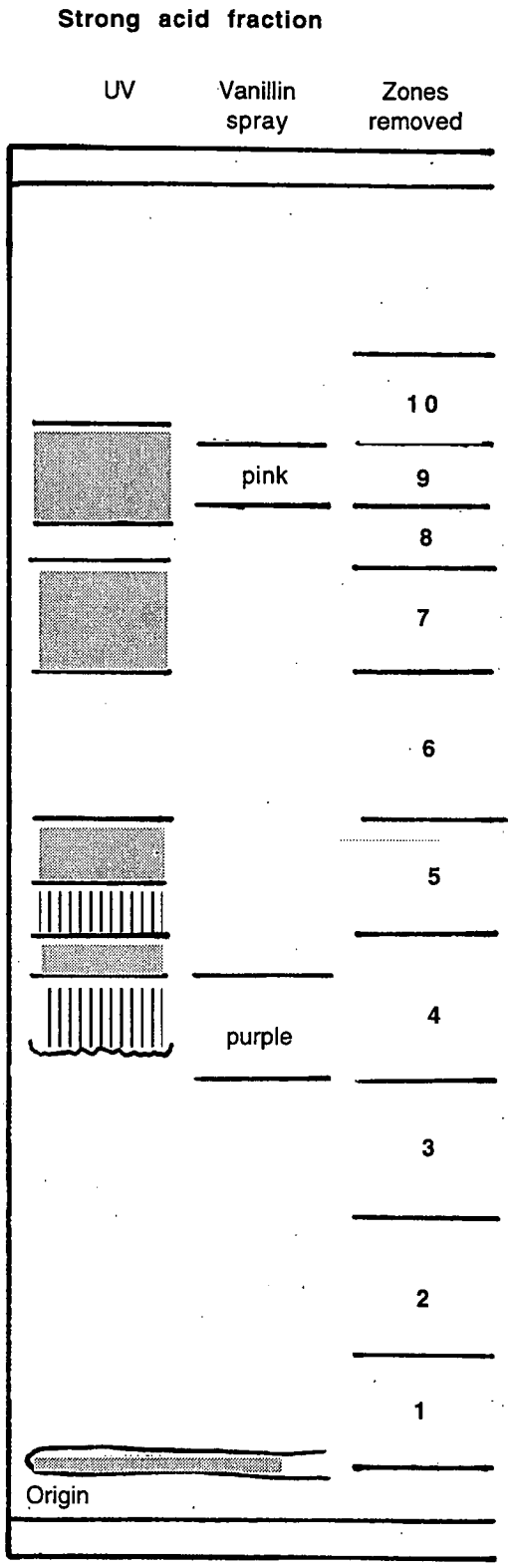


Figure 9.3. Schematic representation of developed pTLC plates for the strong acid fraction of koala urine. The zones removed from each plate are indicated on the right hand side. pTLC conditions are described in section 9.2.4.2. Ci 13 was located in Zones 5 and 6.

The Ci 13 residue from pTLC was dissolved in 5 ml of methanol, diluted with 50% mobile phase (water/methanol 55:45), filtered (0.45 micron nylon filter) and 200 µl injected onto the column. Four fractions were collected accounting for all HPLC peaks. A sample (2 ml) of each fraction was acidified to pH 1 with 5 M HCl and extracted with 1 ml ethyl acetate and the content analysed by GC/MS. Ci 13 was eluted in the final fraction (Figure 9.4).

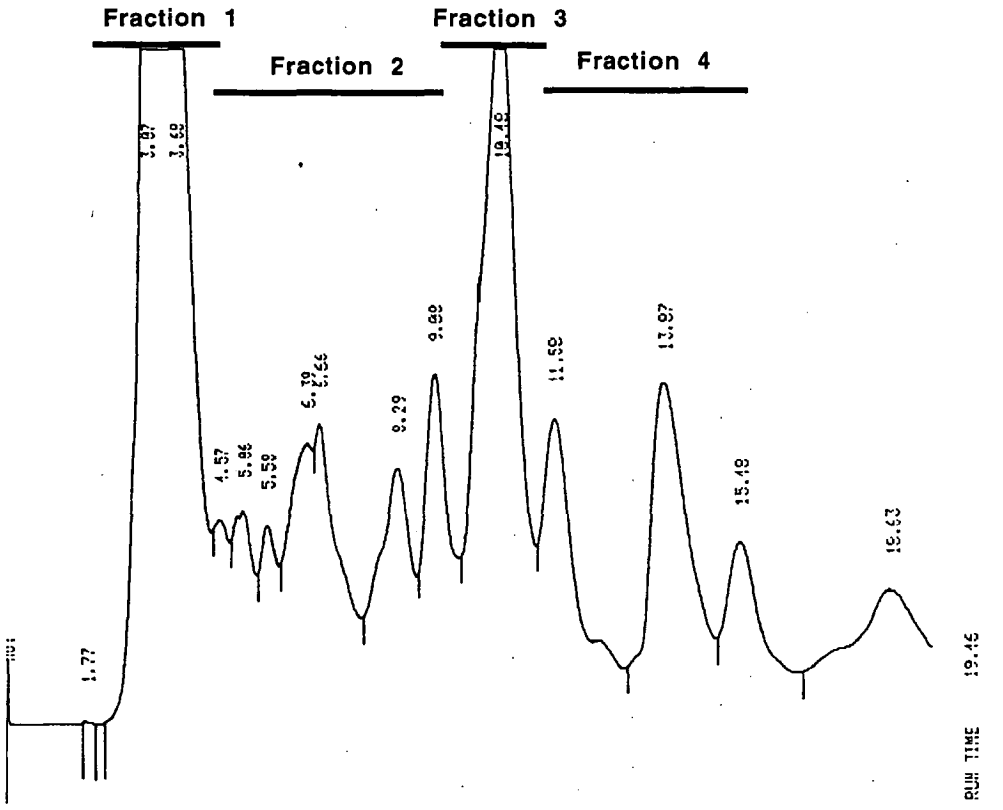


Figure 9.4. pHPLC chromatogram in the final purification of Ci 13 from the strong acid fraction of koala urine. pHPLC conditions are described in section 9.2.4.2. Note that Ci 13 elutes in fraction 4 and although it accounts for approximately 85 % of the sample, it does not produce a significant peak at a wavelength of 214 nm.

The corresponding fractions of a further two pHPLC runs were combined in a large flask and the volume reduced to approximately 300 ml on a rotary evaporator. The remaining aqueous solution was acidified with 5 M HCl and extracted with 3 washes of ethyl acetate. The ethyl acetate extract was then evaporated to dryness. The residue yielded 105 mg Ci 13 (purity, by GC, was 96 %).

9.2.5. Calibration curves for 1,8-cineole metabolites

Calibration curves for five metabolites were prepared. These metabolites were Ci 3 and 5 (9- and 7-hydroxycineole), Ci 2 and 4 (9 and 7-cineolic acid) and Ci 13 (7-

hydroxy-9-cineolic acid). Since it was not practical to isolate all metabolites, these calibration curves were used to quantify structurally similar metabolites for which no standard was available. For example, the calibration curve prepared for Ci 13 was used to quantify all hydroxy cineolic acids. This method was based on the assumption that metabolite isomers will be extracted similarly, and the observation that the molar FID response to a substance is proportional to the number of carbon atoms present (Jorgensen *et al.* 1990).

Two groups of metabolites, the dihydroxycineoles and dicineolic acid, did not have representative metabolites isolated and quantitation of these metabolites used the closest structural calibration curve. Generally these were minor metabolites and an estimation was satisfactory for the purpose of studying the metabolite pattern. The dihydroxycineoles were quantified using the calibration curve prepared for 9-hydroxycineole and Ci 22 was quantified using the calibration curve prepared for Ci 9-cineolic acid. The hydroxy cineolic acids Ci 19 and 20 did not accept a TMS group during derivatisation with BSTFA. Therefore, these two metabolites were quantified separately using a calibration curve of Ci 13 that had been methylated only.

Stock solution of metabolites. Each metabolite, or metabolite mix, was accurately weighed into an autosampler vial and sealed with a teflon/rubber septum and screw cap. The metabolites were diluted with 400 µl of methanol using a micro syringe.

Five dilutions of the stock solution were prepared covering the concentration ranges shown in Table 9.4. The dilutions were made using a micro syringe and adding to each of six tapered centrifuge tubes 10, 20, 30, 40, 50 and 50 µls respectively (the sixth tube being a control tube for monitoring the progress of saponification). The volumes in tubes 1 to 4 were then made to 50 µl with methanol.

Saponification. As in the production of the *p*-cymene metabolite calibration curves, it was necessary to hydrolyse methylated standard metabolites to return them to their excreted state. The standard metabolites could then be subject to the same assay procedure as genuine urine samples.

NaOH (250 µl 0.2 M) was added to each dilution and the tubes were heated at 40°C. Aliquots were taken from the control tube at intervals and analysed by GC to monitor hydrolysis progress. The absence of the derivatised peaks from the chromatogram after 24 h confirmed complete hydrolysis.

Finally, 250 µl of control brushtail possum urine and internal standard (0.5 mg 2,5-dimethylbenzoic acid in 50 µl methanol) was added to each dilution. The dilutions where then acidified, extracted and derivatised as described in Section 9.1.

At least one set of calibration dilutions were analysed with each batch of urine samples. Comparisons of all calibration curves are summarised in Table 9.4.

Table 9.4. Calibration curve concentrations and parameters for 1,8-cineole metabolites quantified in the brushtail possum and koala.

		Ci 2	Ci 4	Ci 3	Ci 5	Ci 13
Stock soln conc[1] (µmol/400µl)		89.8	8.1	98.7	16.6	167.8
Dilution #	Stock soln µl			µmol/0.5ml		
1	0	0.00	0.00	0.00	0.00	0.00
2	10	2.25	0.20	2.47	0.42	4.20
3	20	4.49	0.41	4.94	0.83	8.39
4	30	6.74	0.61	7.40	1.25	12.59
5	40	8.98	0.81	9.87	1.66	16.78
6	50	11.23	1.02	12.34	2.08	20.98
n (calibration curves)		3	3	3	3	3
Equations (mean)[2]		y=0.200x	y=0.748x	y=0.418x	y=0.229x	y=0.331x
Metabolite/IS range		0 - 2.27	0 - 0.88	0 - 5.62	0 - 0.53	0 - 7.48
R^2(mean)		0.99	0.98	0.99	0.97	0.99
CV % (slopes)		5.7	3.9	3.6	3.5	7.3

[1]Dilutions of stock solutions were added to 0.5 ml of diluted urine.
[2]Calibration curve y-intercepts = "0".

9.2.6. Faecal metabolite analyses

9.2.6.1. Brushtail possum faecal analysis

All available faecal pellets from the 1,8-cineole feeding Experiment 2 (see Chapter 10 - section 10.3) were analysed quantitatively for 1,8-cineole metabolites.

A total of twenty four daily collections of faecal pellets were ground separately in a mortar and pestle and weighed. Approximately 1 g (accurately weighed) of faecal grindings were measured into a large centrifuge tube and 10 ml of distilled water added. Tubes were placed on a rotary mixer for 5 h and then left standing overnight to ensure complete breakdown and dissolution of ground faeces.

Approximately 500 mg aliquots of faecal slurry were accurately weighed into clean centrifuge tubes with internal standard (0.5 mg 2,5-dimethylbenzoic acid in 50 µl methanol). The slurry was hydrolysed by adding 200 µl of 1.1 M acetate buffer and 50 µl of extract of *Helix pomatia* and heating at 37.5°C overnight.

Six samples of faecal slurry were also analysed for free metabolite (days 5 and 6 of Experiment 2). These were analysed in the same manner as the hydrolysed samples after incubation.

Faecal samples were acidified to pH 1 with 3 drops of 5 M HCl. Metabolites were extracted into three washes (1 x 2 ml and 2 x 1 ml) of dichloromethane/*n*-propanol 80:20. A sample of the dichloromethane/*n*-propanol extract was evaporated to dryness and redissolved in 500 µl ethyl acetate and methylated with 1 ml

diazomethane and finally 20 µl methylated extract was TMSed with 20 µl of BSTFA.

Faecal extracts were analysed by GC/MS using the same instrument and conditions as those described for urine analyses in section 9.2.2. Calibration curves for quantitation of faecal metabolites were the same as those prepared for urine quantitation.

9.2.6.2 Koala faecal analysis

Faeces were analysed in the course of studying the fate of *p*-cymene in the koala (Chapter 7 - section 7.2.8). Faecal pellets from two animals were analysed for the three days of the *p*-cymene dosing experiment. The analysis of these extracts will be considered in Chapter 11.

9.2.7. Urinary glucuronic acid analysis

Urinary glucuronic acid assays were performed by Mrs Sue Brandon, University of Tasmania. Concentrations were measured directly for each possum and koala urine sample used for urine metabolite analyses. The method is described in Chapter 2 - section 2.2.6. Possum urines were diluted one hundred-fold for the lower concentrations of dietary 1,8-cineole and two hundred-fold for higher concentrations. Koala urines were diluted two hundred-fold and where the glucuronic acid concentrations were high, analyses were repeated with four hundred-fold dilutions. Calibration curves were prepared for each batch of urine analyses.

The method described by Blumenkrantz and Asboe-Hansen (1973) instructs that the absorbance of the reaction mixture should be read within 5 min of adding *m*-hydroxy diphenyl. However, it was observed that the UV absorbance did not stabilise until at least 5 mins after the *m*-hydroxy diphenyl was added, after which it remained stable for at least 30 min.

9.3. Results

9.3.1. Identification of metabolites

Evidence supporting identification of both known and novel 1,8-cineole metabolites was obtained from direct comparison of urine extracts from pre- and post-ingestion of 1,8-cineole in brushtail possums, EI GC/MS, CI GC/MS and high resolution GC/MS analysis of urine extracts, LC/MS of diluted urine and NMR data for the isolated koala metabolite Ci 13. Table 9.3 summarises the assigned names for all metabolites identified in both species. The type of derivative, diagnostic ion used for extracting mass chromatograms and the conversion factor for calculating the TIC from diagnostic ion abundances for each metabolite are also reported in this table.

9.3.1.1. Gas chromatography mass spectrometry

Methylation of urinary metabolites was insufficient for elucidation of the many polar metabolites. Figure 9.1(A and B) demonstrates the improvement in chromatography after trimethylsilylation. The large number of metabolites excreted by brushtail possums is evident in Figure 9.1-B.

A sample of a GC/MS chromatogram of koala urine extract is shown in Figure 9.5.

Figure 9.6 shows the chemical structures of 1,8-cineole metabolites identified. Of the twenty metabolites identified detailed structures of only six are known and structures have been tentatively assigned to a further four. Although specific isomeric structures of the unknown metabolites were not elucidated, the functional groups for each compound were derived from their empirical formulas, molecular weights and mass spectra. The EI mass spectra for individual derivatised metabolites are reported in Table 9.5. Results from CI mass spectrometry and empirical formulas derived from accurate mass data are reported in Table 9.6.

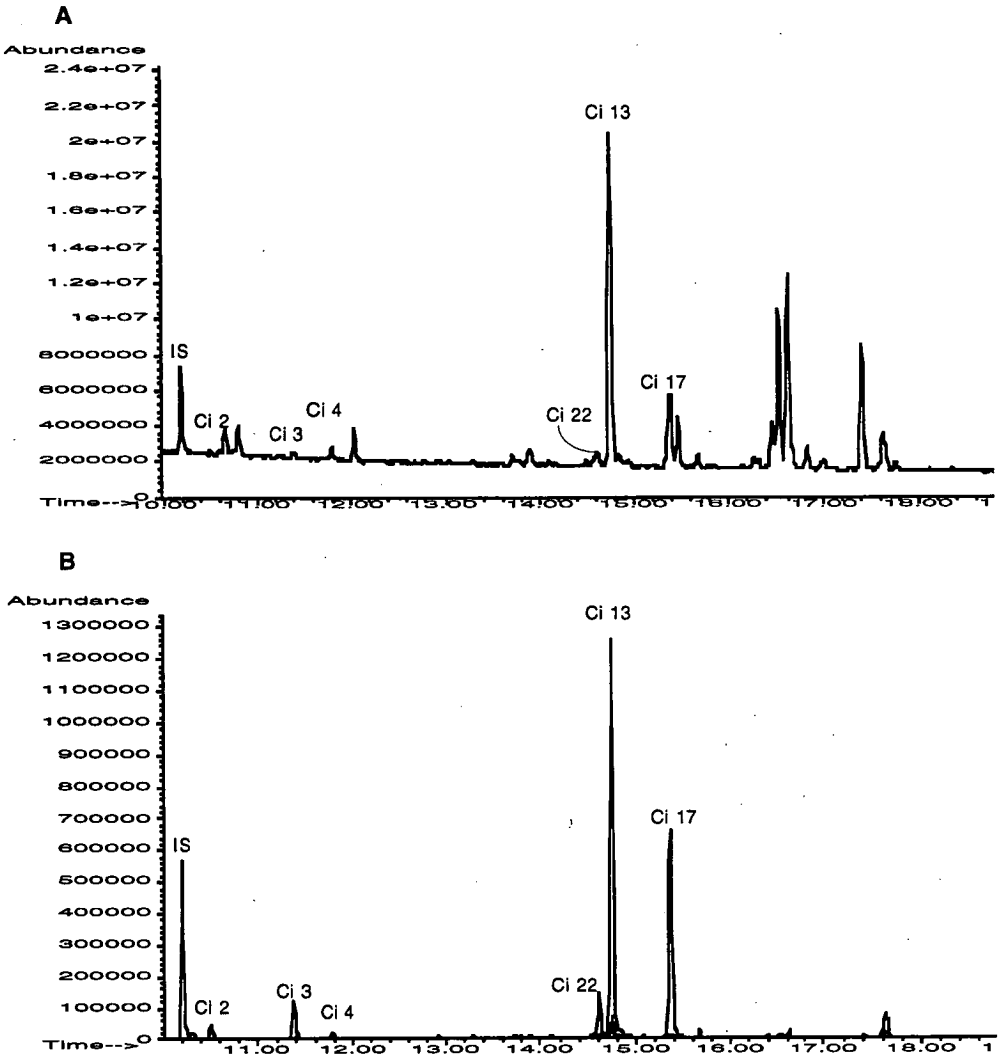


Figure 9.5. A) TIC chromatogram from GC/MS of hydrolysed koala urine extract (methylated and TMSed). B) Mass chromatogram of diagnostic 1,8-cineole metabolite ions (ions at *m/z* : 164, 139,183 and 227) to highlight metabolites. GC/MS conditions described in section 9.2.2. Metabolites are labelled with assigned names. IS = internal standard (2,5-dimethyl benzoic acid). See Table 9.3 and Figure 9.6 for metabolite names and chemical structures

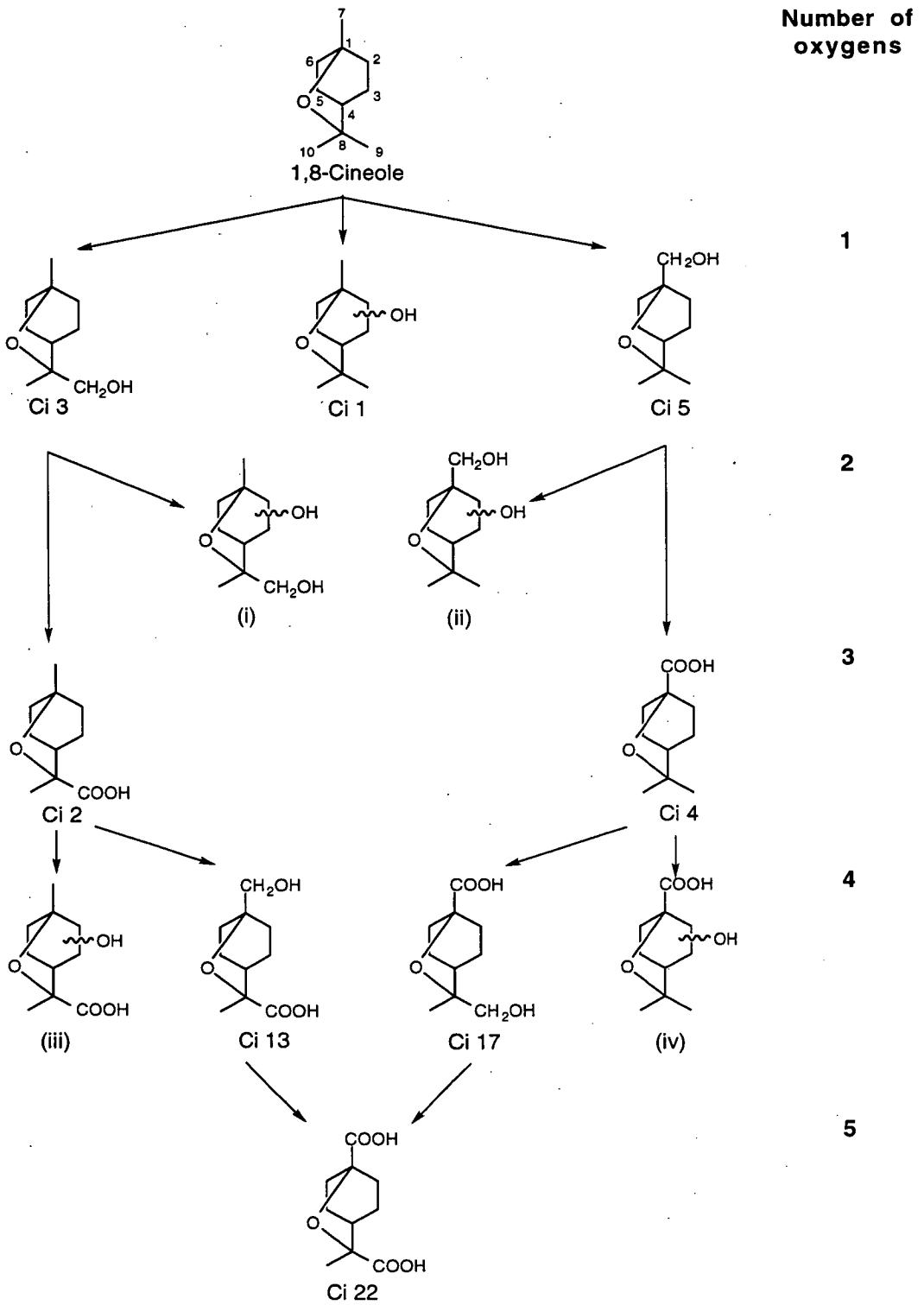


Figure 9.6. Chemical structure of 1,8-cineole and its metabolites. Metabolites are grouped according to the degree of oxidation (1 - 4; see section 9.3.1.4). Known metabolite structures are: Ci 2, 9-cineolic acid; Ci 3, 9-hydroxycineole; Ci 4, 7-cineolic acid; Ci 5, 7-hydroxycineole; Ci 13, 7-hydroxy-9-cineolic acid. The following structures have also been tentatively assigned: Ci 17, 9-hydroxy-7-cineolic acid and Ci 22, 7,9-dicineolic acid. Hydroxy cineolic acids (Ci 6, 7, 8, 9, 12, 15, 18, 19 and 20) whose structures are unkown are isomers of iii and iv. The structures of the dihydroxycineoles (Ci 10 and 11) are also unknown but are isomers of i and ii.

Table 9.5. EI mass spectra for all 1,8-cineole metabolites in both brushtail possums and koalas.

Metabolite[1]	Rt[2] GC/MS	Significant EI ions (m/z) *Relative abundance (%)*																													
Ci 1	10.38	242	227	199	169	160	159	157	152	143	137	130	129	117	111	109	108	101	93	83	77	75	73	71	69	67	59	55	47	45	43
		21	*34*	*11*	*13*	*13*	*100*	*32*	*20*	*37*	*28*	*13*	*16*	*25*	*17*	*24*	*45*	*10*	*45*	*13*	*12*	*44*	*53*	*13*	*15*	*10*	*11*	*12*	*11*	*23*	*70*
Ci 2	10.49	198	140	139	95	71	67	59	53	43	41																				
		tr	*10*	*100*	*32*	*9*	*7*	*3*	*4*	*52*	*6*																				
Ci 3	11.37	140	139	121	95	93	81	75	73	71	67	45	43	41																	
		11	*100*	*4*	*24*	*7*	*5*	*10*	*14*	*7*	*5*	*7*	*63*	*5*																	
Ci 4	11.42	184	183	155	152	151	140	139	123	111	108	95	83	81	80	79	69	67	59	55	53	45	43	41							
		11	*100*	*2*	*5*	*37*	*23*	*33*	*21*	*18*	*11*	*12*	*17*	*27*	*19*	*25*	*30*	*12*	*16*	*26*	*12*	*34*	*54*	*35*							
Ci 5	11.64	242	227	199	183	169	159	143	139	137	135	111	109	107	103	95	94	93	91	84	81	79	75	73	69	67	59	55	45	43	41
		65	*81*	*21*	*100*	*20*	*20*	*44*	*38*	*45*	*33*	*45*	*15*	*25*	*23*	*14*	*16*	*52*	*14*	*24*	*11*	*37*	*60*	*60*	*16*	*16*	*10*	*25*	*20*	*44*	*36*
Ci 6	13.28	228	227	183	143	138	137	111	109	95	93	75	73	59	45	43															
		12	*65*	*39*	*12*	*11*	*93*	*12*	*11*	*19*	*97*	*29*	*46*	*13*	*31*	*100*															
Ci 7	13.71	228	227	183	143	138	137	111	109	95	93	75	73	59	45	43															
		11	*57*	*8*	*13*	*11*	*100*	*11*	*12*	*15*	*94*	*25*	*35*	*10*	*27*	*89*															
Ci 8	13.77	286	254	244	243	228	227	211	153	138	137	117	116	111	109	101	95	93	75	73	59	45	43								
		2	*16*	*12*	*65*	*24*	*100*	*11*	*13*	*11*	*14*	*11*	*55*	*21*	*22*	*61*	*24*	*21*	*42*	*62*	*15*	*25*	*79*								
Ci 9	14.06	227	138	137	131	109	95	94	93	75	73	45	43																		
		50	*12*	*100*	*13*	*16*	*10*	*10*	*99*	*29*	*56*	*28*	*66*																		
Ci 10	14.12	228	227	138	137	117	116	111	109	101	95	93	75	73	59	45	43														
		22	*100*	*11*	*24*	*11*	*47*	*18*	*17*	*48*	*14*	*21*	*26*	*40*	*11*	*17*	*40*														
Ci 11	14.43	227	225	183	157	156	143	137	95	93	75	73	45	43																	
		100	*13*	*12*	*11*	*19*	*10*	*12*	*11*	*16*	*22*	*46*	*10*	*21*																	

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Table 9.5. continued.

Metabolite	Rt[1] GC/MS	Significant EI ions (m/z) *Relative abundance (%)*																												
Ci 12	14.55	286	271	227	197	181	169	149	143	138	137	137	129	117	111	109	107	101	95	93	83	79	75	73	67	59	55	45	43	39
		1	*98*	*100*	*34*	*11*	*10*	*10*	*20*	*12*	*44*	*55*	*20*	*10*	*24*	*10*	*10*	*13*	*13*	*41*	*14*	*19*	*42*	*71*	*12*	*22*	*12*	*28*	*53*	*22*
Ci 13	14.73	271	227	211	137	119	109	94	93	91	79	75	73	59	45	43														
		11	*23*	*7*	*68*	*12*	*11*	*10*	*100*	*15*	*13*	*44*	*49*	*20*	*33*	*54*														
Ci 14[3]	14.78	271	227	209	195	157	156	145	137	105	95	93	75	73	45	43														
		tr	*100*	*16*	*36*	*11*	*26*	*21*	*14*	*15*	*15*	*18*	*33*	*60*	*17*	*36*														
Ci 15	14.84	227	210	196	195	156	145	120	105	93	91	79	77	75	73	45	43													
		34	*45*	*21*	*100*	*11*	*54*	*26*	*38*	*15*	*13*	*9*	*10*	*36*	*69*	*20*	*36*													
Ci 17	15.35	183	151	123	79	75	73	45	43																					
		100	*50*	*23*	*11*	*8*	*14*	*35*	*23*																					
Ci 18	15.42	227	138	137	133	109	93	75	73	45	43																			
		46	*11*	*84*	*10*	*10*	*100*	*27*	*57*	*26*	*21*																			
Ci 19	12.95	156	155	139	138	127	111	110	109	96	95	93	71	67	55	43														
		12	*91*	*17*	*16*	*14*	*30*	*19*	*22*	*13*	*50*	*27*	*10*	*14*	*15*	*100*														
Ci 20	13.03	155	137	95	93	55	45	43																						
		42	*49*	*15*	*68*	*10*	*15*	*100*																						
Ci 22	14.62	183	151	123	81	79	77	45	43																					
		100	*59*	*28*	*17*	*18*	*13*	*53*	*41*																					

[1]Mass spectra are reported for metabolites in their fully derivatised state. Metabolites and their derivatisations are reported in Table 9.3 and chemical structures for underivatised metabolites are shown in Figure 9.6. tr = trace.

[2]Retention times are based on the GC/MS conditions and capillary column described in Section 9.2.1.

[3]Ci 14 was not quantified as it was detectable in only very small amounts in some possums.

Table 9.6. Protonated molecular ions, $[M+H]^+$, accurate mass data, empirical formulae and molecular weights (MW) for 1,8-cineole metabolites derived from CI and high resolution GC/MS.

Metabolite	Derivative[1]	CI-MS[2]		Calc. mass[4]	Empirical	Underivatised
		$[M+H]^+$	Accurate mass[3]	$[M+H]^+$	formulae	MW[5]
Group 1 - hydroxycineoles						
Ci 1	TMS	243	243.1779	243.178	$C_{13}H_{26}SiO_2$	170
Ci 3	TMS	243	243.1749		$C_{13}H_{26}SiO_2$	170
Ci 5	TMS	243			$C_{13}H_{26}SiO_2$	170
Group 2 - cineolic acids						
Ci 2	M	199	199.1336	199.1334	$C_{11}H_{18}O_3$	184
Ci 4	M	199	199.1328		$C_{11}H_{18}O_3$	184
Group 3 - dihydroxycineoles						
Ci 10	TMS	331	331.2145	331.2125	$C_{16}H_{34}Si_2O_3$	186
Ci 11	TMS	331			$C_{16}H_{34}Si_2O_3$	186
Ci 14	TMS	331			$C_{16}H_{34}Si_2O_3$	186
Group 4 - hydroxy cineolic acids						
Ci 6	M + TMS	287	287.1682	287.1679	$C_{14}H_{26}SiO_4$	200
Ci 7	M + TMS	287			$C_{14}H_{26}SiO_4$	200
Ci 8	M + TMS	287			$C_{14}H_{26}SiO_4$	200
Ci 9	M + TMS	287			$C_{14}H_{26}SiO_4$	200
Ci 12	M + TMS	287			$C_{14}H_{26}SiO_4$	200
Ci 13	M + TMS	287	287.17092		$C_{14}H_{26}SiO_4$	200
Ci 15	M + TMS	287			$C_{14}H_{26}SiO_4$	200
Ci 17	M + TMS	287			$C_{14}H_{26}SiO_4$	200
Ci 18	M + TMS	287			$C_{14}H_{26}SiO_4$	200
Ci 19	M	215			$C_{11}H_{18}O_4$	200
Ci 20	M	215			$C_{11}H_{18}O_4$	200
Group 5 - dicineolic acid						
Ci 22	M	243			$C_{12}H_{18}O_5$	214

[1]Methyl ester (M) and/or TMS ether (TMS).
[2]CI-MS conditions are described in the text Section 9.2.3.
[3]Accurate mass data were obtained from at least one metabolite in each group.
[4]Calc. mass = calculated mass (to four decimal points).
[5]Underivatised metabolites are inferred from CI-MS $[M+H]^+$.

9.3.1.2 Liquid chromatography-mass spectrometry (LC/MS)

Results from this method supported the mass spectral findings from GC/MS. It was particularly useful as MS and MS/MS data for underivatised metabolites confirmed the presence and identity of metabolites and their glucuronides. Further identification of metabolites was not possible by this method without reference standards. Nor was it possible to directly reconcile metabolites identified by GC/MS to those detected by LC/MS as the different modes of mass spectrometry produce incomparable mass spectra due to their respective characteristic sensitivities. It is interesting to note that LC/MS detected two dihydroxycineole glucuronides in the koala which were not detected by GC/MS (Chapter 11).

Positive ion APCI MS and MS/MS data for each metabolite are reported in Table 9.7. and Table 9.8. for possums and koalas, respectively. The metabolites frequently

formed adducts with the mobile phase components (water and methanol) and these are also reported. For some metabolites the adducts produced the major ion and MS/MS data were therefore reported for the adduct. The hydroxy cineolic acids in the koala urine also formed dimers at high concentrations.

The positive ion APCI LC/MS chromatogram for the brushtail possum was very complex (Figure 9.7). The complexity of the TIC MS chromatogram was improved by extracting mass chromatograms of diagnostic ions. This effectively highlighted the peaks associated with each group of metabolites.

Although the 1,8-cineole metabolite profile of koala urine is relatively simple compared to the brushtail possum, the positive ion APCI LC/MS chromatogram has some minor metabolites being overwhelmed by other peaks (Figure 9.8). Once again extracting mass chromatograms of diagnostic ions highlighted the metabolite peaks for each metabolite group.

Since a reverse phase HPLC column was used for the chromatography, the retention times could be used as a measure of metabolite polarity, with the earlier eluting metabolites being the most polar. This would indicate that some glucuronide metabolites are less polar than the dihydroxycineole and the hydroxy cineolic acid metabolites.

MS/MS of the MH^+ of 1,8-cineole metabolites resulted in similar fragmentation patterns. Free metabolites frequently lost water and produced a characteristic 1,8-cineole ion (eg. *m/z*'s 139 or 155). Serial losses of water were characteristic for glucuronides. Glucuronides also commonly lost the ion *m/z* 176. A similar pattern was also observed in non-1,8-cineole glucuronides in the koala urine.

Table 9.8 also reports the MS/MS of non 1,8-cineole derived MH^+s in koala urine in an attempt to identify glucuronide conjugates. Negative ion ESI provided further information on the non 1,8-cineole glucuronides. Figure 9.9 shows the TIC chromatograms for both unhydrolysed and hydrolysed urine samples. Peaks A to F disappeared with hydrolysis, confirming them to be glucuronides. Extracting mass chromatograms again highlighted these peaks in the lower chromatograms. Table 9.9 reports the MS and MS/MS data for these glucuronides.

Mass spectral evidence of the dicineolic acid metabolite was also obtained by the negative ion ESI LC/MS. It was not detectable by positive ion LC/MS.

The molecular weights of the glucuronide aglycones suggests they are small aromatic and phenolic molecules such as benzoic acid, methyl phenol, benzyl alcohol, salicylic acid, methyl catechol, methyl resorcinol and methyl hydroxyquinol.

Table 9.7. Positive ion APCI LC/MS and MS/MS data for 1,8-cineole metabolites in brushtail possum urine (diluted 1:10). Figure 9.7 shows the chromatogram associated with the peaks in this table. Urine was collected on day 8 of 1,8-cineole feeding experiment 1 (diet containg 2 % 1,8-cineole - see Chapter 10).

Rt[1] LC/MS	Metabolite group[2]	MS *(m/z)* [3]			MS/MS (*m/z*) of major MS ion[4]				
		MH^+	$M+H_2O$	MH^++CH_3OH					
Glucuronides									
3.95	3*	**363**	380		345 MH^+-H_2O	187 MH^+-gluc	169 MH^+-gluc-H_2O	151 MH^+-gluc-$2H_20$	
4.75	3*	**363**	380		345 MH^+-H_2O	327 MH^+-2H_2O	309 MH^+-3H_2O	187 MH^+-gluc	151 MH^+-gluc-$2H_20$
6.58	3*	**363**	380		345 MH^+-H_2O	327 MH^+-2H_2O	309 MH^+-3H_2O	187 MH^+-gluc	151 MH^+-gluc-$2H_20$
5.32	4*		**394**		No MS/MS data as metabolite co-elutes with a stronger peak				
13.14	2*		**378**		202 $(M+H_2O)$-gluc	185 $(M+H2O)$-gluc-H_2C	139 cineole ion		
15.84	1*	**347**			329 MH^+-H_2O	311 MH^+-2H_2O	293 MH^+-3H_2O	171 MH^+-gluc	
16.63	1*	**347**			329 MH^+-H_2O	311 MH^+-2H_2O	293 MH^+-3H_2O	171 MH^+-gluc	
17.44	1*	**347**			329 MH^+-H_2O	311 MH^+-2H_2O	293 MH^+-3H_2O	171 MH^+-gluc	

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Table 9.7. continued.

Rt[1] LC/MS	Metabolite group[2]	MS (*m/z*) [3]			MS/MS (*m/z*) of major MS ion[4]		
		MH^+	$M+H_2O$	$MH^+ + CH_3OH$			
Free metabolites							
6.05	4	**201**	218	233	183 MH^+-H_2O	165 MH^+-2H_2O	155 cineole ion
8.00	4	**201**	218		No MS/MS data as metabolite co-eluted with a stronger peak		
9.78	4	**201**			183 MH^+-H_2O	165 MH^+-2H_2O	155 cineole ion
11.09	4	201	**218**	233	201 $(M+H_2O)-H_2O$	165 $(M+H_2O)-3H_2O$	155 cineole ion
12.40	4	**201**	218	233	183 MH^+-H_2O	165 MH^+-2H_2O	155 cineole ion
18.93	2	**185**	202		139 cineole ion		
19.24	2	**185**	202		167 MH^+-H_2O	139 cineole ion	
18.23	1	**171**	188		153 MH^+-H_2O	135	
19.86	1	**171**			153 MH^+-H_2O	135	

[1]Rt from HPLC conditions described in text (Section 9.2.3). Diluted neat urine was used - therefore metabolites are underivatised.

[2]1* = hydroxy cineole glucuronide, 2* = cineolic acid glucuronide, 3* = dihydroxycineole glucuronide, 4* = hydroxy cineolic acid glucuronide, 1 = hydroxy cineole, 2 = cineolic acid and 4 = hydroxy cineolic, gluc = glucuronic acid.

[3]Mass spectra of water or methanol adducts or metabolite dimers were common. In some cases these were the major ions and MS/MS data was acquired for these ions. The ion from which MS/MS data originated is highlighted in bold.

[4]Daughter ion fragmentation patterns are explained where possible. Similar patterns of fragmentation recurred, especially for the glucuronides where multiple water ions were lost.

Table 9.8. Positive ion APCI LC/MS and MS/MS data for 1,8-cineole metabolites in koala urine (diluted 1:10). Figure 9.8 shows the chromatogram associated with the peaks in this table. Urine was from koalas feeding on *E. cephalocarpa* (from *p* -cymene dose 0.37 mmol/kg on day 2 - see Chapter

Rt[1] LC/MS	Metabolite[2] group	MS *(m/z)* [3] MH^+	$M+H_2O$	$MH^+ + CH_3OH$	MH^+ dimer	MS/MS (*m/z*) of major MS ion[4]				
Cineole derived peaks										
Glucuronides										
3.85	3*	363	**380**			363 $(M+H_2O)-H_2O$	345 (M+H2O)-2H2O	187 $(M+H_2O)$-gluc		
6.26	3*	363	**380**			363 $(M+H_2O)-H_2O$	345 (M+H2O)-2H2O	187 $(M+H_2O)$-gluc		
7.33	4*		**394**			218 $(M+H_2O)$-gluc	201 $(M+H_2O)$-gluc-H_2O	183 $(M+H_2O)$-gluc-$2H_2O$	165 $(M+H_2O)$-gluc-$3H_2O$	
13.06	2*		**378**			202 (M+H2O),-gluc	185 $(M+H_2O)$-gluc-H_2O	139 cineole #		
15.82	1*	**347**	364			329 MH^+-H_2O	311 MH^+-2H_2O	293 MH^+-3H_2O	171 MH^+-gluc	153 MH^+-gluc-H_2O
Free metabolites										
11.02	4	201	**218**	233	401	201 $(M+H_2O)-H_2O$	183 (M+H2O)-2H2O	165 (M+H2O)-3H2O		
12.06	4	201	**218**	233	401	201 $(M+H_2O)-H_2O$	183 (M+H2O)-2H2O	165 (M+H2O)-3H2O	155 cineole ion	
Non-cineole derived peaks										
9.50	B glucuronide MW 300		**318**			301 $(M+H_2O)-H_2O$	283 $(M+H_2O)-2H_2O$	265 $(M+H_2O)-3H_2O$	247 $(M+H_2O)-4H_2O$	125 $(M+H_2O)$-gluc
9.71	C glucuronide MW 300		**318**			301 $(M+H_2O)-H_2O$	283 $(M+H_2O)-2H_2O$	265 $(M+H_2O)-3H_2O$	247 $(M+H_2O)-4H_2O$	125 $(M+H_2O)$-gluc
7.04	Hippuric acid	**180**			359	162 MH^+-H_2O	105 $C_7H_5O_1$			

[1,2,3,4] See Table 9.7. footnotes for interpretations.

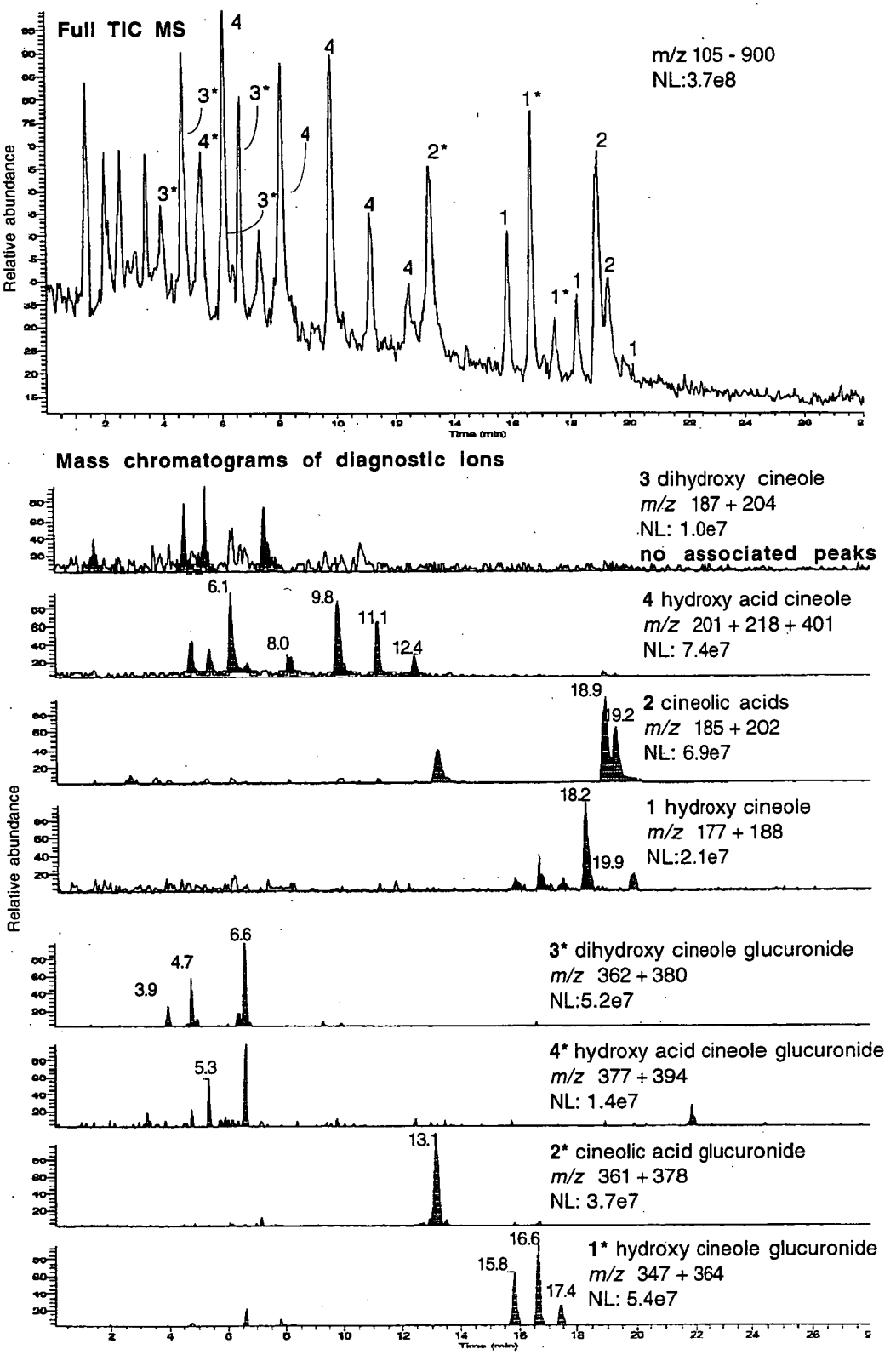


Figure 9.7. TIC chromatograms from positive ion APCI LC/MS of unhydrolysed possum urine (top). Mass chromatograms of diagnostic ions highlight each metabolite group (group 1 to 4 and their respective glucuronides indicated by * - see Section 9.3.1.4 for metabolite group summaries; bottom). Metabolite groups, diagnostic ions and normalisation levels (NL; indicating relative size of peaks) are recorded for each chromatogram. Metabolites peaks are labelled with retention times on mass chromatograms and are identified on the TIC chromatogram.

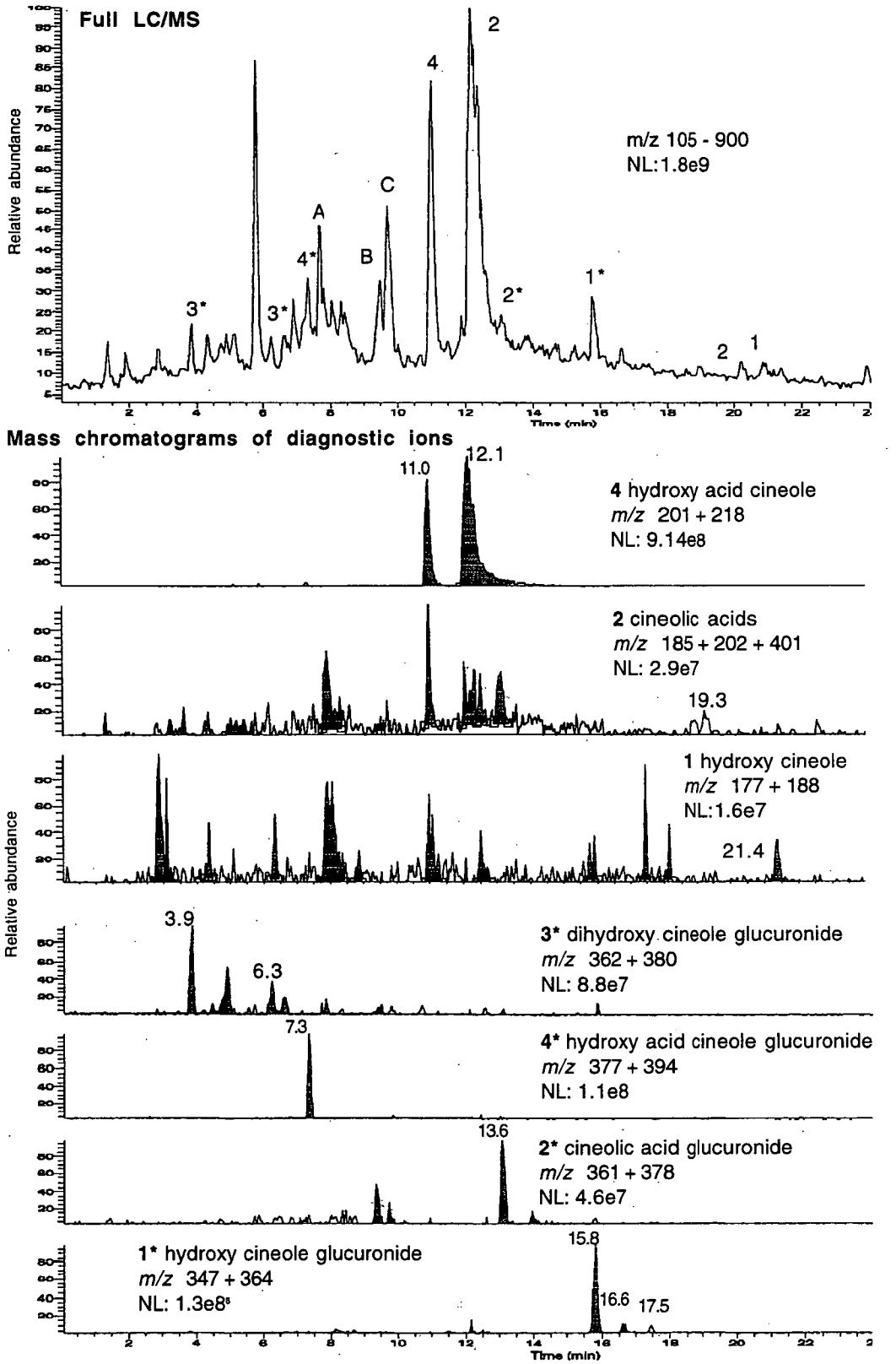


Figure 9.8. TIC chromatograms from positive ion APCI LC/MS of unhydrolysed koala urine (top). Mass chromatograms of diagnostic ions highlight each metabolite group (groups 1 to 4 and their respective glucuronides indicated by * ; bottom). Metabolite group, diagnostic ions and normalisation levels (NL) are recorded on each chromatogram. Metabolites are depicted by retention times on the mass chromatograms and identified on the TIC chromatogram, along with unknown glucuronides (A, B and C; see Figure 9.9).

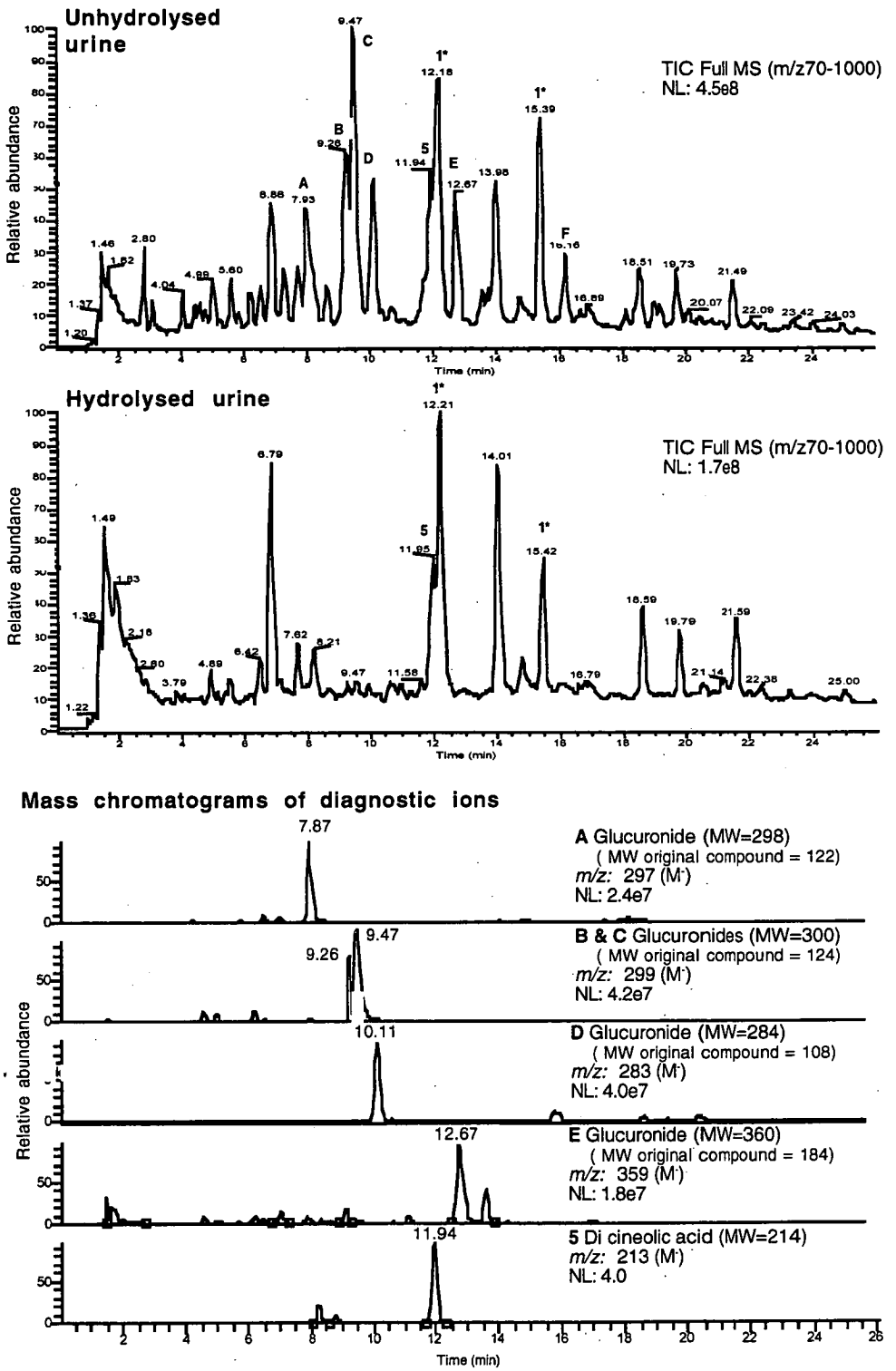


Figure 9.9. TIC MS chromatograms from negative ion ESI LC/MS for unhydrolysed and hydrolysed koala urine. Mass chromatograms of diagnostic ions highlight glucuronide compounds (A - E) which disappeared after hydrolysis. Compounds A - F are likely to be small aromatic and phenolic molecules. Normalisation levels (NL) indicate the relative strength of each chromatogram. Retention times are marked on the mass chromatograms for each compound. Negative ion ESI MS identified the dicineolic acid metabolite (group **5** - see Section 9.3.1.4) which is highlighted the bottom mass chromatogram. Hydroxycineole glucuronides (**1***) are the only other 1,8-cineole metabolite identified in this mode of LC/MS and are marked on the TIC chromatogram.

Table 9.9. Negative ion electrospray LC/MS and MS/MS data for 1,8-cineole metabolites in koala urine (diluted 1:10). Urine was from koalas feeding on *E. cephalocarpa* (see Chapter 11). Figure 9.9 shows the chromatogram associated with the peaks in this table.

Rt[1] LC/MS	Metabolite[2] group	MS *(m/z)* [3] M⁻	M⁻ dimer	MS/MS[4] *m/z*		Molecular weight Glucuronide	Aglycone[5]
Cineole derived peaks							
11.94	5	**213**		169 M⁻-COO			214
Non-cineole derived peaks							
7.87	A	**297**		175 gluc⁻	121 aglycone⁻	298	122
9.26	B	**299**	599	175 gluc⁻	123 aglycone⁻	300	124
9.47	C	**299**	599	175 gluc⁻	123 aglycone⁻	300	124
10.11	D	**283**	567	175 gluc⁻		284	108
12.67	E	**359**	719	183 aglycone⁻	175 gluc⁻	360	184
16.16	F	345	**691**	Dimer major MS ion - therefore no MS/MS data			170

[1]Rt from HPLC conditions described in text (Section 9.2.3).
[2]5=dicineolic acid, A - F = unknown glucuronides.
[3]Metabolites often formed dimers. The dimer was the major ion for compound F.
The MS/MS results are from the dominant MS ion, highlighted in bold.
[4]gluc⁻ = glucuronic acid (negative ion).

9.3.1.3 NMR of Ci 13

^{1}H NMR, 399.714 MHz, $CDCl_3$ δ (ppm): 3.66 (s 3H, -OC**H**$_{c3}$); 3.33 (pseudoquartet[AB] 2H, $\mathbf{H_a}$); 3.05 (broad s 1H, -O**H**); 1.93 (m 1H, $\mathbf{H_g}$); 1.87 (m 1H, $\mathbf{H_m}$); 1.83 (m 1H, $\mathbf{H_f}$); 1.66 (m 1H, $\mathbf{H_e}$); 1.52 (m 1H, $\mathbf{H_j}$); 1.49 (m 2H, $\mathbf{H_k}$ $\mathbf{H_l}$); 1.31 (s 3H, C$\mathbf{H_{b3}}$); 1.29 (m 1H, $\mathbf{H_h}$); 1.28 (m 1H, $\mathbf{H_j}$). See Figure 9.10-A for positions of labelled protons.

^{13}C NMR, 100.575 MHz, $CDCl_3$ δ (ppm): 177.23 (**C**=O); 78.88 ($\mathbf{C_\alpha}$); 73.64 ($\mathbf{C_\beta}$); 67.80 ($\mathbf{CH_{a2}}$); 51.98 (-O$\mathbf{CH_{c3}}$); 30.95 ($\mathbf{CH_m}$); 26.34(m 1H, $H_e\mathbf{CH_h}$); 25.22 ($H_f\mathbf{CH_i}$); 24.28 ($\mathbf{CH_{b3}}$); 22.62 ($H_k\mathbf{CH_l}$); 19.95 ($H_g\mathbf{CH_j}$).

NMR results were interpreted and the structure of Ci 13 was elucidated to be 7-hydroxy-9-cineolic acid. The isomeric structure and relative stereochemistry are shown in Figure 9.10 (A and B respectively). An NOE interaction between the acetoxy methyls and H_m prove this isomeric structure. From the chemical eqivalence of H_k and H_l it could be suggested that the molecule is in a confirmation with these ring protons bent down and away from the deshielding influence of the ether moiety. Energy calculations were performed which showed this to be the energetically preferred stereoisomer.

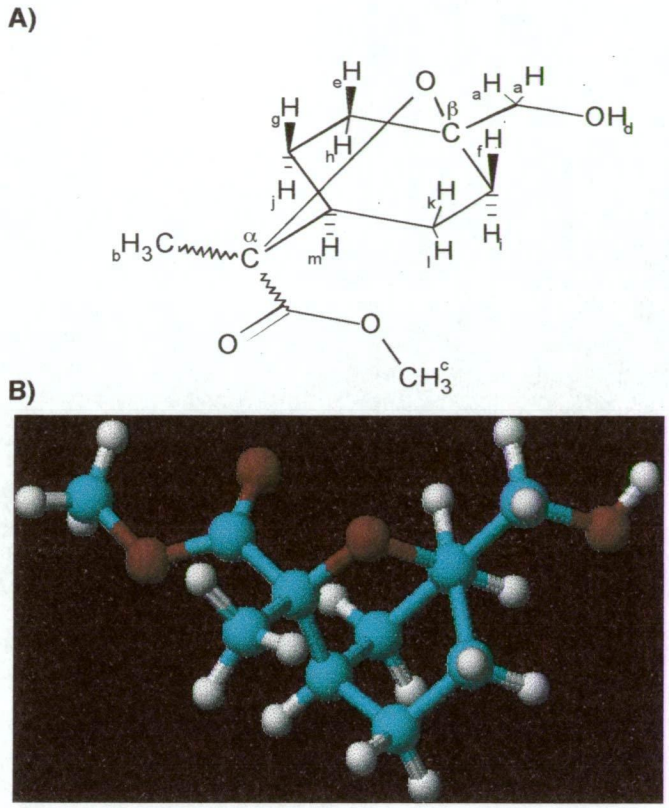


Figure 9.10. A) Isomeric structure and B) relative stereochemistry of Ci 13, 7-hydroxy-9-cineolic acid, interpreted from ^{1}H and ^{13}C NMR.

9.3.1.4 Metabolite groupings

Metabolites were assigned to groups according to the extent of oxidation they had undergone and the functional groups they acquired during oxidation. A summary of the five groups follows.

1) Hydroxycineoles - Ci 3 (9-hydroxycineole), Ci 5 (7-hydroxycineole) and Ci 1. These metabolites acquired a single oxygen to form alcohol and hydroxy metabolites.

2) Cineolic acids - Ci 2 (9-cineolic acid) and Ci 4 (7-cineolic acid) result from further oxidation of 9- and 7-hydroxycineoles respectively. Only two cineolic acid structures are possible.

3) Dihydroxycineoles - Ci 10 and Ci 11 were excreted as minor metabolites in the possum. LC/MS detected dihydroxycineole glucuronides in koala urine, however, they were not detected by GC/MS suggesting they were present in minor amounts in this species. The isomeric positions of the hydroxyl groups were not elucidated.

4) Hydroxy cineolic acids - eleven hydroxy cineolic acids were identified in quantifiable amounts (Ci 6, 7, 8, 9, 12, 13, 15, 17, 18, 19 and 20). More were present in trace amounts in some urine samples (eg. Ci 14). Isomeric structures were assigned to some metabolites in this group, albeit tentatively in some cases. NMR data

confirmed the positions of the carboxyl and hydroxyl groups of Ci 13 as 7-hydroxy-9-cineolic acid (Section 9.3.1.3). Ci 17 was tentatively assigned as 9-hydroxy-7-cineolic acid, based on its EI-MS fragmentation pattern being very similar to that of 7-cineolic acid. This could only result from the carboxylic acid moiety occuring at the C7 position, as it does in 7-cineolic acid, and a β cleavage of the ring, producing the common ion fragment at *m/z* 183. This type of fragmentation is characteristic for most 1,8-cineole metabolites. The C9 with the TMS hydroxyl group would be cleaved and therefore not alter the mass spectrum significantly. The structures of Ci 19 and 20 were also deduced due to the fact that the hydroxy groups were sterically hindered and did not accept a TMS group during derivisation. The most likely carbon position to be sterically hindered is C4, therefore it is proposed that this carbon is the site of hydroxylation in these metabolites. Furthermore, as C7 and C9 are the only carbons that can be oxidised to carboxylic acids, the structures of Ci 19 and 20 are likely to be 4-hydroxy-9-cineolic acid and 4-hydroxy-7-cineolic acid, respectively (based on the relative abundance of Ci 19 and 20 compared to the relative abundances of Ci 3 to 5 and Ci 2 to 4).

5) Dicarboxylic acid - significant amounts of Ci 22 were found in koala urine. Ci 22 was assigned the structure of 7,9-dicarboxylic acid as oxidation to carboxylic acid can only occur on the C7 and 9. Furthermore, the mass spectrum for Ci 22 was again similar to 7-cineolic acid. Therefore by using the same argument of fragmentation used for assigning the structure for Ci 17 (above), the mass spectral evidence confirms the structure of Ci 22. Ci 22 was the most extensively oxidised metabolite identified, acquiring a total of 4 oxygens.

9.3.2. Faecal analysis of 1,8-cineole metabolites

Experimental results of 1,8-cineole recovery in faecal samples are reported in Chapters 10 and 11 for possums and koalas, respectively. A sample GC/MS of hydrolysed faecal extract from brushtail possums is shown in Figure 9.11. The overall extractable content of faeces was much more extensive than urine. The 1,8-cineole metabolites were minor in the overall scheme of the chromatogram. The mass chromatograms of diagnostic ions used to extract 1,8-cineole metabolites revealed a similar metabolite profile in the faecal and urine extracts of possums.

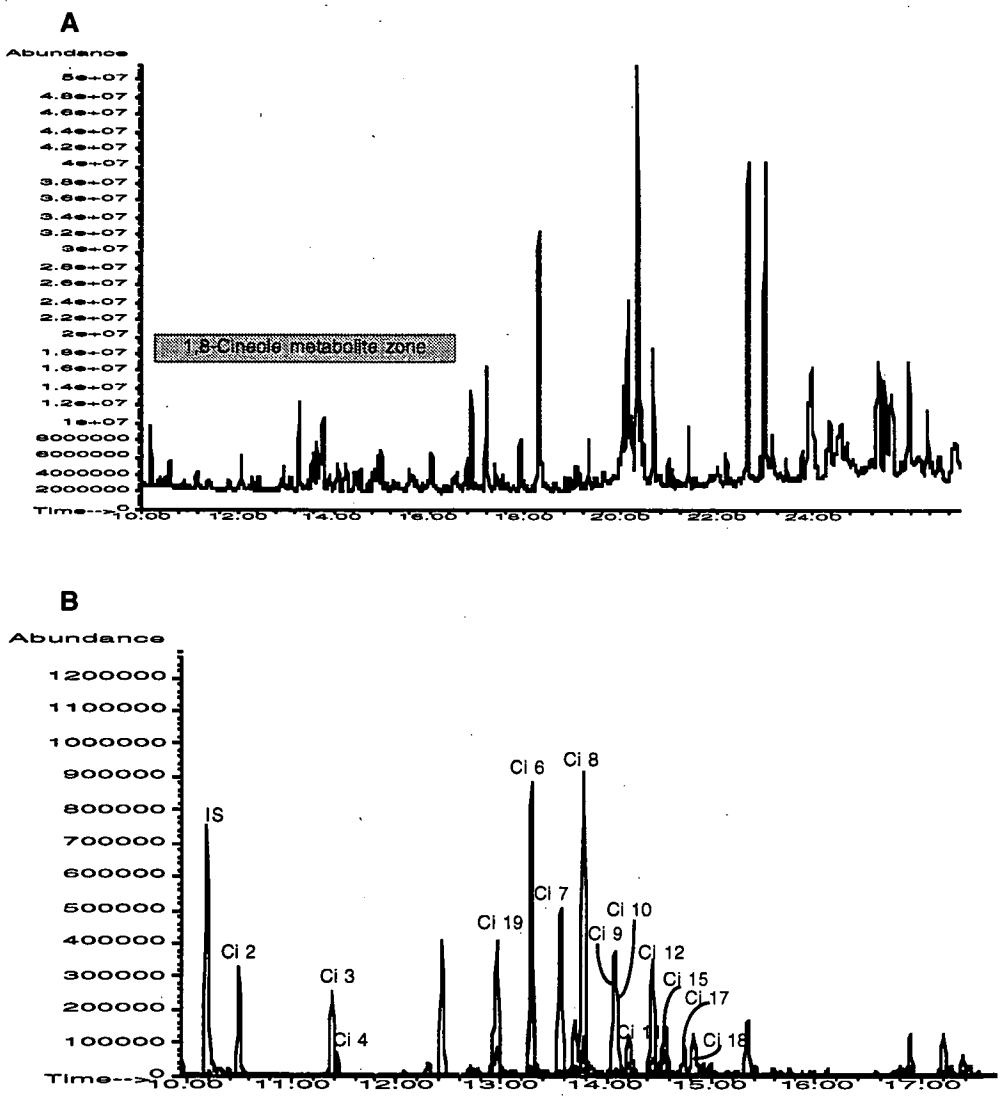


Figure 9.11. A) TIC chromatogram from GC/MS of hydrolysed brushtail possum faecal extract (methylated and TMSed). B) 1,8-Cineole metabolites highlighted by extracting mass chromatogram of diagnostic ions (*m/z*' s at 164, 139, 155, 183 and 227) between 10 and 17 min, covering the retention times of known 1,8-cineole metabolites. GC conditions are described in section 9.2.2. Metabolites are labelled with assigned names. IS = internal standard (2,5-dimethyl benzoic acid). See Table 9.3 for identification of metabolites.

9.3.3. Glucuronic acid quantitation

Experimental results of urinary glucuronic acid concentrations are reported in the relevant chapters.

Fresh calibration curves were prepared for each batch of urine samples analysed. In total 9 calibration curves were prepared. Reproducibility of calibration curves was within acceptable limits. The coefficient of variation for low 20 µg/0.4 ml and high 40 µg/0.4 ml concentration standards were 14.5 and 8.2 % respectively (coefficient

of variation of the slopes derived from the regression equations of all curves was 10.9%).

9.4. Discussion

Identifying and assigning structures of 1,8-cineole metabolites was assisted by the following techniques:

• Direct comparison of urine extracts from pre- and post-ingestion of 1,8-cineole in the brushtail possum. This comparison was not possible in the koala as pre-ingestion samples could not be obtained since koalas are obligate leaf, and therefore terpene, eaters.

• Direct comparison with mass spectra of reference 1,8-cineole metabolites.

• Mass spectral investigation of derivatised metabolites using EI GC/MS, CI GC/MS and high resolution mass spectrometry.

• Mass spectral evidence from LC/MS and MS/MS reflecting the metabolite pattern of 1,8-cineole in unhydrolysed and underivatised urine.

• NMR data for determining the structure of Ci 13.

Elucidation of all 1,8-cineole metabolite structures and their relative stereochemistry represents a major research project in itself. Grouping metabolites according to their degree of oxidation suited the purpose of studying the metabolic fate of 1,8-cineole from an ecological perspective.

We have shown that an extremely complicated array of 1,8-cineole metabolites are excreted by the brushtail possum and koala. In this study 20 metabolites were identified in either the free or glucuronidated form, plus minor amounts of others. The structure of five dihydroxycineoles, excreted by brushtail possums, have been reported in the literature (Carman *et al.* 1994; Carman and Rayner 1994; Carman and Garner 1996). We only detected two, with occasional traces of a third. We were unable to identify the dihydroxycineoles from the mass spectral data reported in the literature since it was necessary to doubly derivatise urine extracts before adequate separation could be achieved by gas chromatography, altering their mass spectra.

The possible combinations for 1,8-cineole oxidation are extensive. The chiral structure of 1,8-cineole ensures that possible isomers from a single oxidation results in multiple stereoisomers. As the number of oxidations increases, the possible combinations of enantiomers and diastereomers escalates dramatically. This occurs to such an extent that a total of 22 stereoisomers are possible just from the further hydroxylation of 9-cineolic acid.

The complexity of stereoisomers was somewhat simplified in our study as the GC capillary column used did not separate enantiomers. A chiral capillary column would be required to separate such compounds, but was beyond the scope and interest of this study. Interestingly, Carman and Klika (1992) reported some preliminary evidence that enantiomeric ratios of 9-hydroxycineole and 9-cineolic acid differ

between male and female possums and suggested they have a role in chemical communication.

Flynn and Southwell (1979) reported the first structural elucidations of 1,8-cineole metabolites. They determined the structure of 9-hydroxy-1,8-cineole and 9-cineolic acid in brushtail possum urine extracts. Since that time a number of other new metabolites have been reported in the possum: 7-hydroxy-1,8-cineole and 7-cineolic acid (Bull *et al.* 1993), 2α,4-dihydroxy-1,8-cineole (Carman and Rayner 1994), 7,9-dihydroxy-1,8-cineole and 2α,7-dihydroxy-1,8-cineole (Carman and Garner 1996) and 2,9-dihydroxy- and 2,10-dihydroxy-1,8-cineole (Carman *et al.* 1994). The fate of 1,8-cineole in insects and microbes has also been studied resulting in the elucidation of other novel metabolites (MacRae *et al.* 1979; Carman *et al.* 1986; Liu and Rosazza 1990; Southwell *et al.* 1995).

Of the twenty metabolites reported in this study, the eleven hydroxy cineolic acids have not been reported as 1,8-cineole metabolites. Combined, these metabolites accounted for the major part of the urinary metabolites in each species (approximately 60 % in the possum and 90 % in the koala). That this group of metabolites has yet to be detailed in the literature is testament to the complexity of 1,8-cineole metabolism. In the decade of detailed research into 1,8-cineole metabolites by Dr Ray Carmans' University of Queensland group, they have only studied the non-acidic urinary metabolites and the previously known simple monoacids (personal communications with Dr Ray Carman, University of Queensland). Furthermore, others may have missed these compounds in the past as they are very polar compounds which required double derivatisation of acid and alcohol moieties before adequate separation was achieved by gas chromatography.

CHAPTER 10

CHRONIC FEEDING EXPERIMENTS OF 1,8-CINEOLE IN BRUSHTAIL POSSUMS

10.1. Introduction

Three experiments investigated chronic feeding of 1,8-cineole in brushtail possums. In each, observational data of dietary intake were combined with detailed analysis of urinary metabolite excretion. This combination provides a thorough and novel insight into the ingestion and excretion of 1,8-cineole in the possums. Observational details of food intake, 1,8-cineole intake, urine volume and pH measurements, faecal output and possum weights were all recorded. Urine and faecal analysis allowed detailed qualitative and semi-quantitative examination of urinary metabolites for selected 1,8-cineole intakes.

The aim of the first experiment was to establish whether 1,8-cineole on its own deters feeding when it is consumed in concentrations that could be expected to be encountered naturally in a *Eucalyptus* diet. The second experiment examined the pattern of urinary metabolites in possums when they were challenged with a high concentration of 1,8-cineole after an extended period of non exposure (ie. naive possums). The final experiment attempted to alter 1,8-cineole intake and metabolism by pretreating possums with a general inhibitor of metabolism and excretion. Analysis of urinary metabolites also provided important information on how the possum metabolises the terpene 1,8-cineole.

Approval for the following feeding trials was granted from the University of Tasmania's Ethics Committee (Animal Experimentation) (Approval number 97015) and the Department of Environment and Land Management, Wildlife Service (Approval number FA 96223).

10.2. Experiment 1 - Threshold intakes of 1,8-cineole

The capacity of possums to consume 1,8-cineole was challenged by a diet containing increasing amounts of 1,8-cineole (0 - 4 % wet weight). A maximum concentration of 4 % was chosen as a comparison to a high oil yield species of *Eucalyptus* with a terpene profile consisting mainly of 1,8-cineole (eg. *E. radiata;* Foley 1992). It was expected that this concentration would challenge the metabolic capacity of possums as it has been shown that possums cannot subsist on a single species of *Eucalyptus* (Freeland and Winter 1975; Dearing and Cork 1999). The aim was to determine whether an intake threshold was reached above which no more 1,8-cineole could be ingested, thereby restricting food intake. Urinary metabolites provided an insight into the metabolism of 1,8-cineole in brushtail possums as well as indicating any saturation of metabolic pathways.

10.2.1. Methods

Animals. Six brushtail possums (weight 3.73 ± 0.82 kg (mean ± sd); 2 females and 4 males; identified as BP 7 to 12) were trapped around the University of Tasmania. They were individually housed in purpose built, covered, outdoor enclosures and were provided with a nest box and aerial branches. Between experiments, possums were maintained on a diet of chopped apples, carrots, lettuce leaf and occasionally bread, dog biscuits and bananas for variety. Possums were weighed regularly and their weights remained constant (3.73 ± 0.82 kg, mean ± sd) throughout their captivity (Appendix 8 - Figure A8.1).

Dosing and urine collection. The possums were transferred to metabolism cages (60 x 60 x 45 cm) at the start of each experiment. The cages were housed in a temperature (20 °C) and light (12 h light/dark) controlled room. Food and water were supplied *ad lib* and daily intake of each measured (control samples for both food and water allowed adjustment of intake due to evaporative losses). Urine was collected and pH and volume measured daily. Metabolism cage floors and collecting funnels were washed with distilled water which was combined with the urine for analyses. A sample of each collection was stored at - 18 °C for later analysis.

Urine was measured at the same time food and water were replenished and this occurred in the early afternoon. Therefore urine analyses correspond to food and 1,8-cineole intakes for the same 24 h period.

Excess urine collected from the higher dietary concentrations of 1,8-cineole was combined and stored at -18°C for isolating metabolites later (Chapter 9 - section 9.2.4.1).

Possums were fed a basal diet, freshly prepared each day, for the duration of the feeding experiments (Table 10.1). A food processor was used to grate the apple, carrot and silver beet and then all ingredients were thoroughly mixed by hand in a bucket. The diet was highly acceptable to the possums.

Table 10.1. Basal diet recipe for brushtail possums used for 1,8-cineole feeding experiments. Quantities are for six possums.

Ingredients	g	% total (wet)
lucerne (chaff ground 1mm mesh)	180	4.5
brown sugar	120	3
carrot (grated)	330	8.2
apple (grated)	1590	39.6
silver beet (grated and chopped)	1200	29.9
water	600	14.9
Total	4020	100

Note: diet is about 16% dry matter

Diet recipe supplied by Dr Clare McArthur, CRC Temperate Hardwood Forests, University of Tasmania

The appropriate daily quantity of 1,8-cineole was incorporated into the diet by adsorbing it onto the lucerne chaff and then mixing the sugar and wet ingredients in order of bulk. The amount of 1,8-cineole added depended on the experimental day. Possums spent two days (days 0 and 1) adjusting to the metabolism cages and were fed a 1,8-cineole free diet. On the third day (day 2), 0.5 % wet weight of 1,8-cineole was mixed into the diet. The 1,8-cineole concentration was increased by 0.5 % wet weight every second day to a maximum concentration of 4.0 % wet weight (days 16 and 17). The diet was divided into seven individual trays (six possums and one control) and weighed before being offered to the possums and again on removal the following day.

Urinary metabolite analyses. Urine samples were analysed as described in Chapter 9 - section 9.2.1. Free and total metabolite levels were measured as well as urinary glucuronic acid. Representative urine samples from three different dietary concentrations of 1,8-cineole were selected for urine analyses. All urine samples from days 3, 9 and 17 (0.5, 2.0 and 4.0 % cineole wet weight) were selected. These days correspond to the second day of each 1,8-cineole concentration.

10.2.2. Results

10.2.2.1. Dietary intake

Figure 10.1 reports the daily intakes of food and 1,8-cineole for all possums (mean ± se). Food intake decreased as the 1,8-cineole concentration increased throughout the experiment. At the higher 1,8-cineole concentrations there was a consistent reduction in food intake for all possums. The correlation between 1,8-cineole and food intakes (Figure 10.1-C) shows an inversely proportional relationship between the intakes of 1,8-cineole and food.

1,8-Cineole intake increased as the dietary concentration increased (Figure 10.1-B). Due to the variability between possums, it was unclear from the mean data whether the apparent threshold of 1,8-cineole ingestion at approximately 3.5 g/kg 1,8-cineole was representative for all possums. Individual possum intakes confirm that BP 7, 8, 11 and 12 demonstrated a threshold in 1,8-cineole ingestion and BP 9 an 10 intakes were variable and inconclusive (see Appendix 8 - Figure A8.2).

Figure 10.2 (A and B) shows the urine volume and pH throughout the experiment. Urine volumes were variable between experimental days. A statistically insignificant trend towards reduced urine output at higher concentrations probably reflects the reduction in food intake. Urine acidity increased progressively as 1,8-cineole ingestion increased (Figure 10.2-B). Faecal output also proved to be highly variable and again a small trend towards reduced output with the higher concentrations of 1,8-cineole was apparent (Figure 10.2-C). At the higher concentrations of 1,8-cineole, possums frequently failed to defaecate, sometimes for a few days at a time.

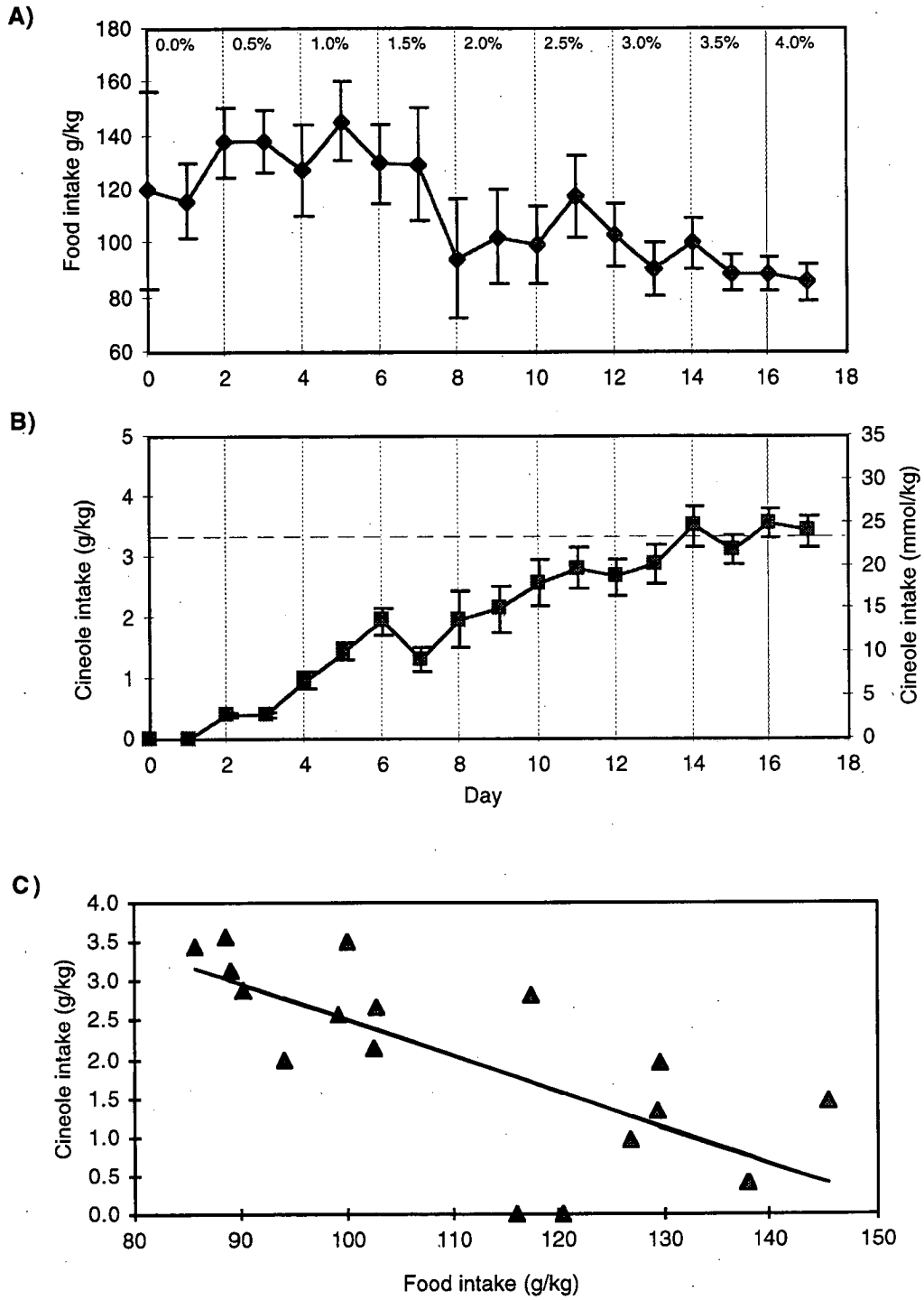


Figure 10.1. A) Food intake (g/kg; mean ± se) decreased as 1,8-cineole concentrations increased throughout Experiment 1 (Anova (single factor) *df* = 16, F = 2.02, *P* = 0.02). B) 1,8-Cineole intakes (g/kg and mmol/kg) for each day (mean ± se) suggest a probable 1,8-cineole intake threshold (dashed line). 1,8-Cineole concentrations (shown on the top graph) were increased every second day in increments of 0.5 %. C) Correlation between 1,8-cineole intake (g/kg) and food intake (g/kg) (*df* = 16, R^2 = 0.56, *P* < 0.001). N = 6 possums in each case.

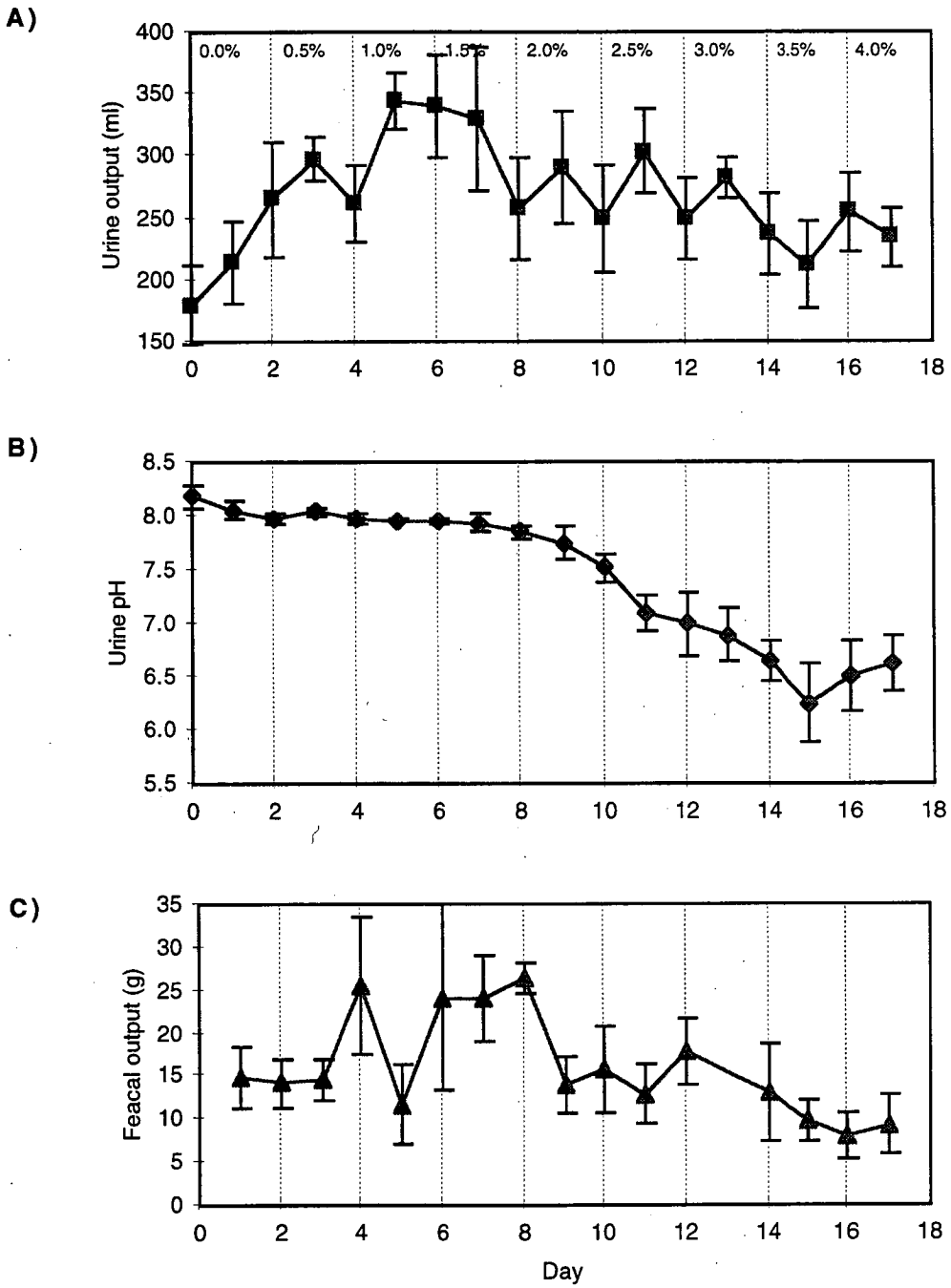


Figure 10.2. Urine and faeces data for each day of Experiment 1. A) Urinary output, B) urine pH and C) weight of faeces produced. Urine pH decreased significantly with increasing 1,8-cineole intake (Anova (single factor) df = 16, F = 11.97, P < 0.001). Although not of statistical significance, there was a trend suggesting a reduction in urine and faeces production as the 1,8-cineole concentration increased (Anova (single factor) df = 16, F = 1.26, P = 0.24 and df = 16, F = 1.51, P = 0.12 for urine and faeces, respectively). Values are reported as mean ± se, n=6. Concentrations of 1,8-cineole are noted on the top panel.

10.2.2.2. Metabolite analyses

The dietary concentration of 1,8-cineole and corresponding intake for the days when urine samples were analysed are summarised in Table 10.2. Full data, including metabolite analyses, on individual possums are reported in Appendix 8 - Table A8.1.

Table 10.2. 1,8-Cineole dietary concentrations (% wet weight) and quantity of 1,8-cineole ingested on the days for which urinary metabolites were analysed in Experiment 1.

	Day 3		Day 9		Day 17	
	Mean	sd	Mean	sd	Mean	sd
1,8-Cineole concentration						
1,8-cineole	0.5		2.0		4.0	
1,8-Cineole intake						
gm	2.4	0.5	5.5	2.2	12.2	3.0
mmol	15.8	3.5	35.4	14.0	79.0	19.2
mmol/kg	4.5	1.0	10.3	4.4	22.3	4.1

1,8-Cineole-derived metabolites accounted for the majority of peaks occurring in the gas chromatogram of derivatised urine extract (see Chapter 9 - Figure 9.1). Eighteen metabolites were identified in quantifiable amounts in the urine. These metabolites encompassed a range of oxidation states and were grouped according to the degree of oxidation (see Chapter 9 - section 9.3.1.4). Each metabolite group was well represented, except for the dicarboxylic acid which was only found in the koala. There were three hydroxycineole metabolites, two cineolic acids, two dihydroxycineoles and eleven hydroxy cineolic acids. The amounts of individual metabolites measured for each urine sample day are shown in Figure 10.3. There was little variability in the pattern of metabolite excretion as 1,8-cineole concentration increased.

The recovery of ingested 1,8-cineole as both free and total metabolites for each sample day is reported in Table 10.3. More metabolite was recovered after hydrolysis. The fractional recovery of free and total metabolites did not vary between the three sample days. Conjugation of individual metabolites will be detailed later in this section.

Because of the large number of metabolites excreted they were grouped, to facilitate the identification of patterns of metabolism, as follows: 1) hydroxycineoles, 2) cineolic acids, 3) dihydroxycineoles and 4) hydroxy cineolic acids (Chapter 9 - section 9.3.1.4).

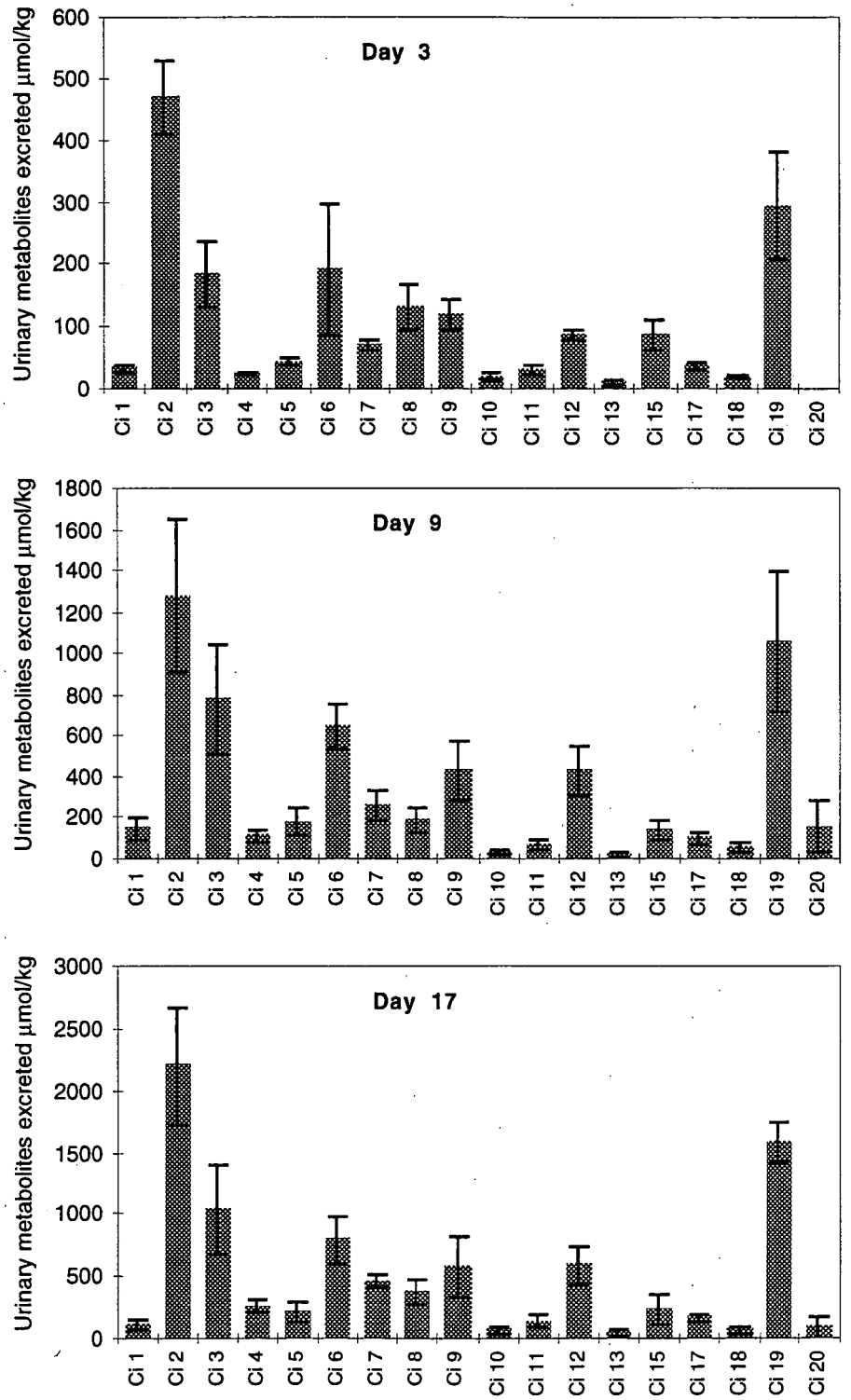


Figure 10.3. Individual 1,8-cineole metabolite recoveries (µmol/kg), after hydrolysis, for each day metabolites were analysed in Experiment 1 (mean ± se). Metabolites are identified in Chapter 9 - Figure 9.6 and Table 9.3.

Table 10.3. Fractional recoveries of 1,8-cineole intakes as urinary metabolites in Experiment1.

Possum	Day 3		Day 9		Day 17	
	Free	Total	Free	Total	Free	Total
Mean ± sd	0.31 ± 0.11	0.44 ± 0.14	0.37 ± 0.15	0.55 ± 0.21	0.23 ± 0.10	0.40 ± 0.16

Metabolite recoveries increased after hydrolysis for each day (Anova (two factor with repl.) *df* = 1, F = 10.3 *P* = 0.003). The free and total fraction of metabolites recovered did not change across the three days (Anova (single factor) *df* = 2, F = 2.17, *P* = 0.15 and *df* = 2, F = 1.15, *P* = 0.34 for free and total recoveries respectively).

The excretion of metabolite groups, after hydrolysis, is shown in Figure 10.4, as the percent of urinary metabolites on a molar basis. This minimises the effect of variation between 1,8-cineole intake and fractional recoveries. Overall, the hydroxy cineolic acid metabolites accounted for approximately 60 % of the total (hydrolysed) excreted metabolites. Cineolic acids were the next most abundant group (~25 %), then hydroxycineoles (~13 %) and dihydroxycineoles accounted for the remaining small percentage of metabolites.

Figure 10.4 also shows that the relative proportion of each group did not vary significantly with increasing intakes of 1,8-cineole.

The extent of hydrolysable conjugation for each metabolite group is shown in Figure 10.5. Hydrolysis increased the recovery of hydroxycineoles, cineolic acids and consequently the total metabolite recovery. Conjugation of dihydroxycineoles and hydroxy cineolic acids was not significant.

Free and total molar recoveries for individual metabolites are shown in Figure 10.6 and the associated statistical comparisons are reported in Table 10.4. Individual metabolites tended to reflect the same pattern of conjugation as their overall group. The least oxidised compounds, the hydroxycineoles, underwent the greatest degree of conjugation. The cineolic acids were the next most extensively conjugated. The dihydroxycineole metabolites Ci 10 and 11 did not undergo any significant conjugation. Finally, although there was no significant conjugation of the hydroxy cineolic acids as a group, minor conjugation of Ci 6, 7 and 18 was found. The molar recovery of free Ci 20 was greater than the total recovery. This anomaly may be due to a deterioration in the chromatography of Ci 20 since the hydroxyl group on Ci 20 did not form a TMS derivative. Therefore the methyl ester of Ci 20 was more polar than any of the other fully derivatised metabolites.

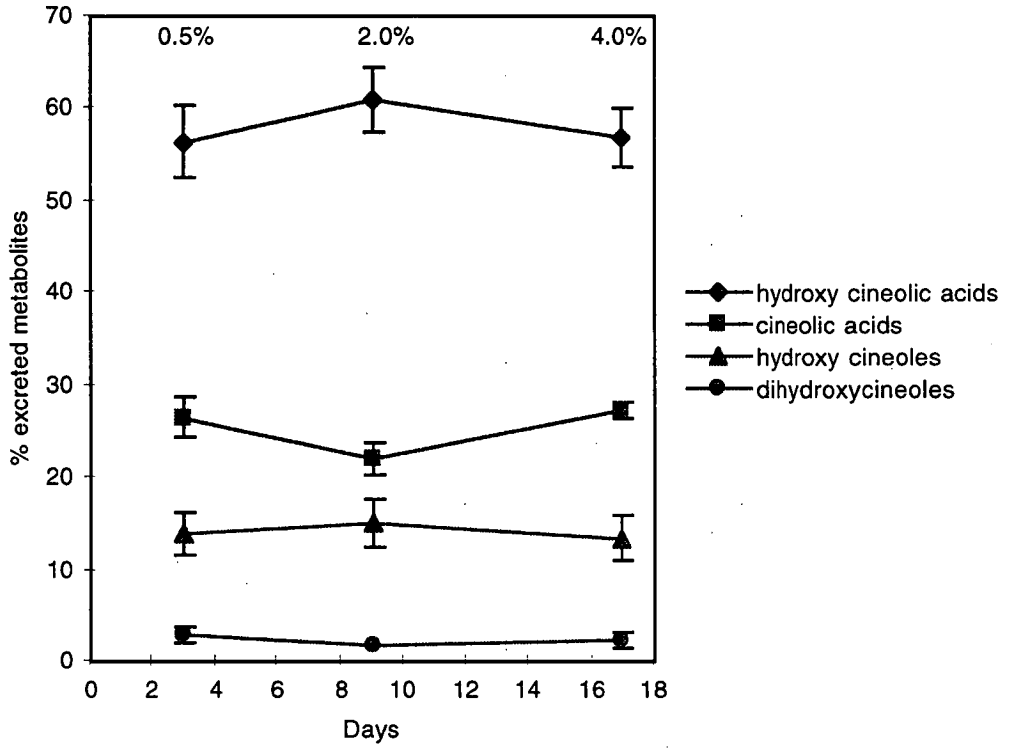


Figure 10.4. Recovery of 1,8-cineole metabolite groups as a percentage of total urinary metabolites for brushtail possums in Experiment 1 (mean ± se, n = 6). The dietary 1,8-cineole concentrations for respective urine sample days are shown at the top of the figure. The relative proportion of each group did not change significantly as the 1,8-cineole concentration increased (Anova (single factor) for 1) hydroxycineoles *df* = 2, F = 0.11, *P* = 0.90, 2) cineolic acids *df* = 2, F = 0.2.71, *P* = 0.10, 3) dihydroxycineoles *df* = 2, F = 0.78, *P* = 0.47 and 4) hydroxy cineolic acids *df* = 2, F = 0.54, *P* = 0.59).

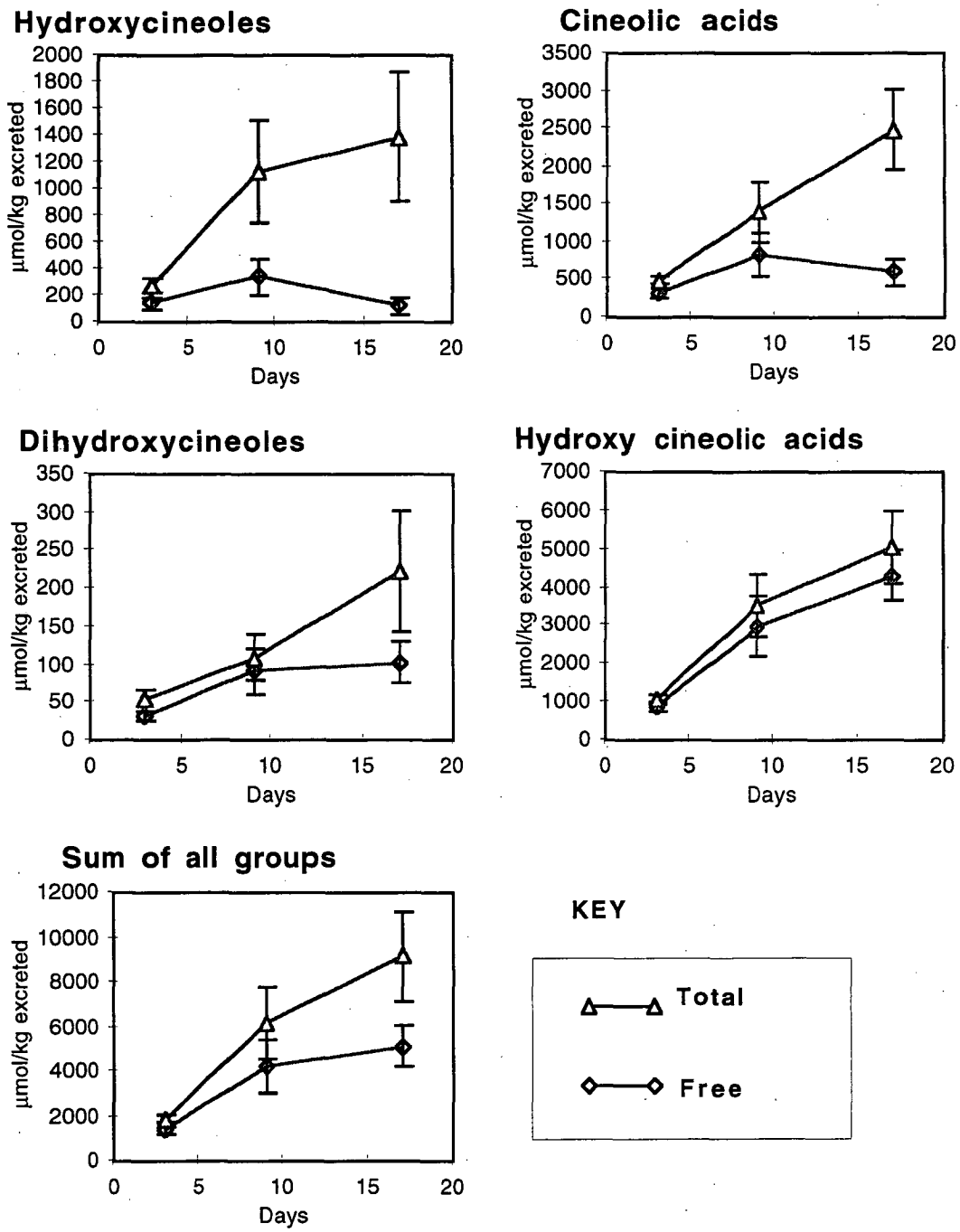
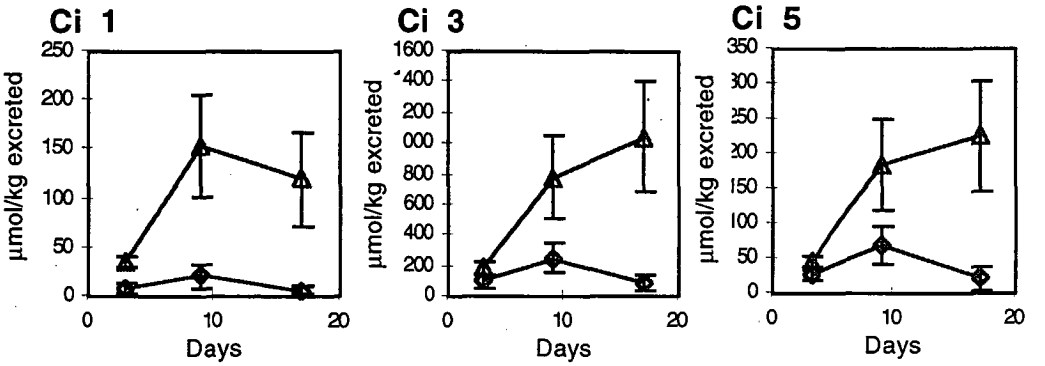
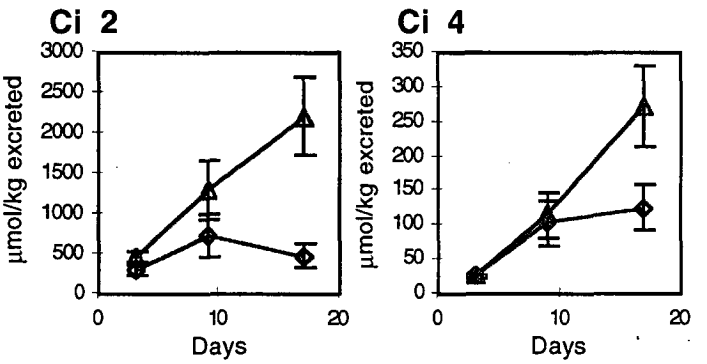


Figure 10.5. Comparison of free and total metabolites for each metabolite group. Values are µmol/kg (mean ± se, n = 6). Hydrolysis increased the recovery of hydroxycineoles, cineolic acids and total metabolite recovery (Anova (two factor with replication) df = 1, F = 11.75, P = 0.002; df = 1, F = 12.11, P = 0.002 and df = 1, F = 4.80, P = 0.04, respectively), but did not affect the dihydroxycineole group (df = 1, F = 2.54, P = 0.10).

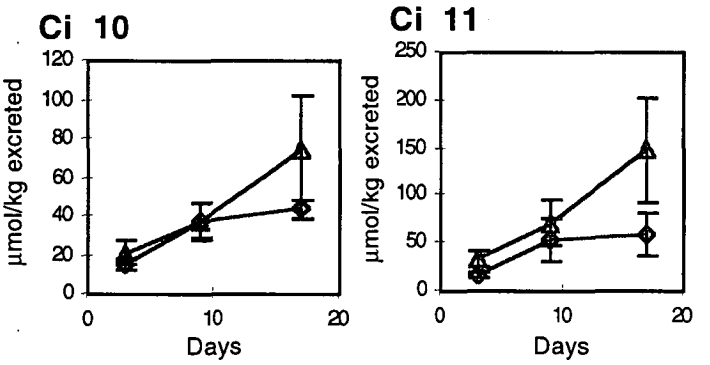
1) Hydroxycineoles



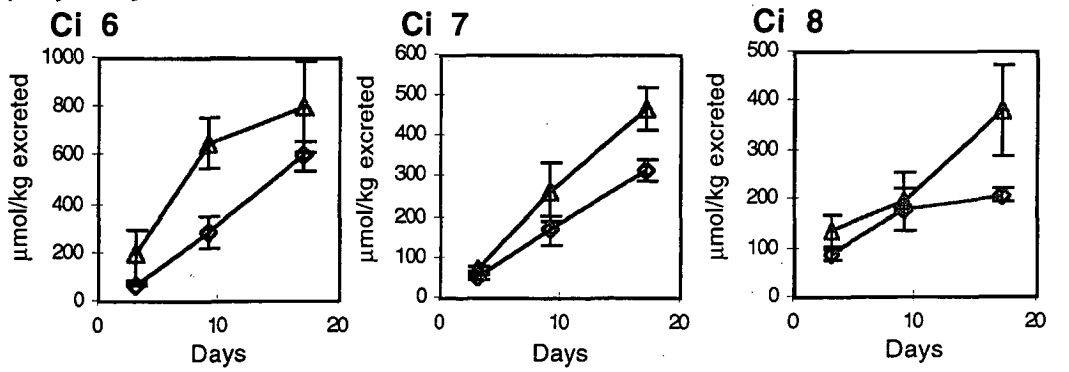
2) Cineolic acids



3) Dihydroxycineoles



4) Hydroxy cineolic acids



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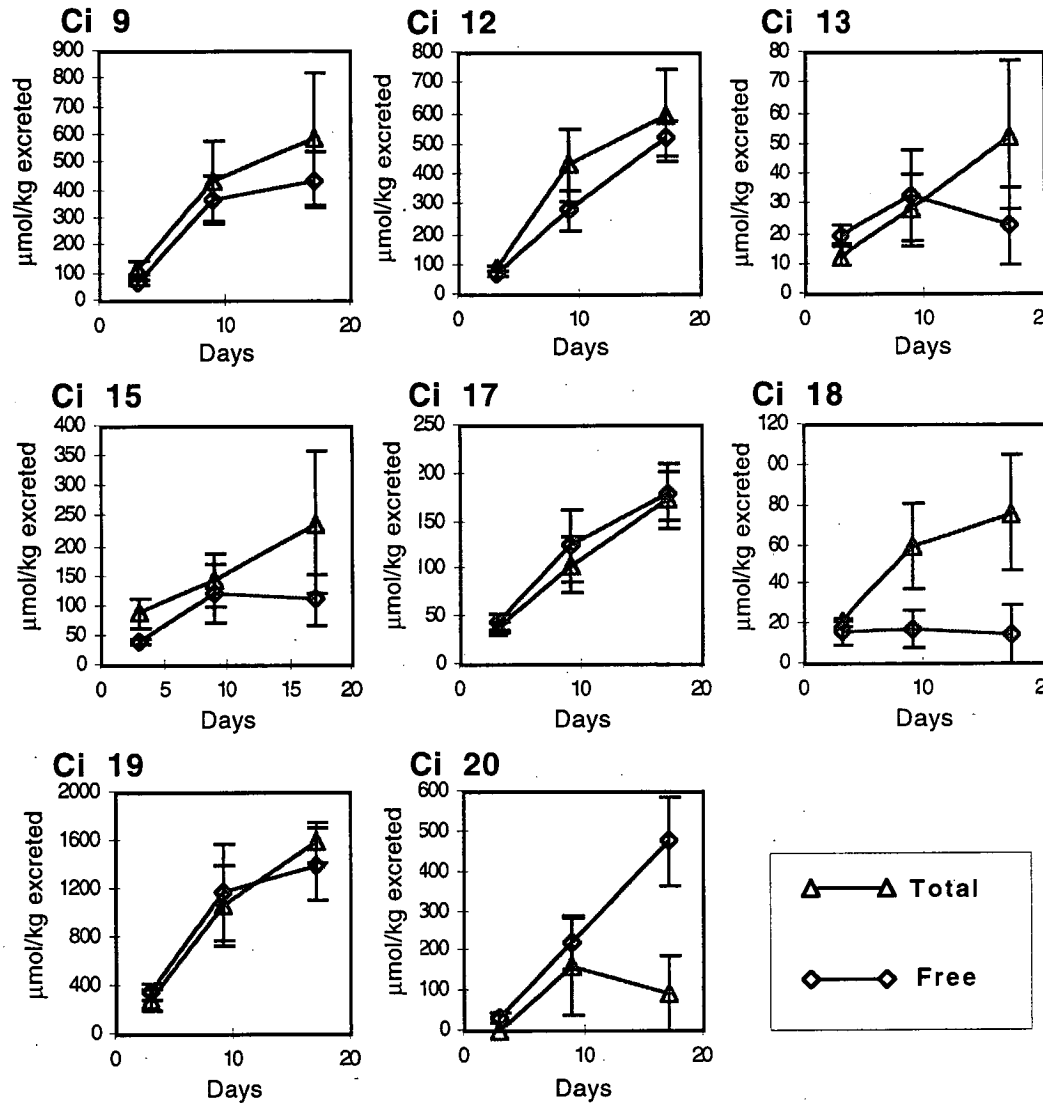


Figure 10.6. Free and total recoveries of individual metabolites. Values are µmol/kg (mean ± se, n = 6). Anova comparisons are reported in Table 10.4.

Table 10.4. Statistical comparisons of total and free recoveries for the individual metabolites shown in Figure 10.6.

Metabolite	Anova two factor with replication (n = 6)		
	df	F value	*P* value
Hydroxy cineoles			
Ci 1	1	13.79	<0.001
Ci 3	1	11.19	0.002
Ci 5	1	9.81	0.004
Cineolic acids			
Ci 2	1	11.71	0.003
Ci 4	1	3.81	0.06
Dihydroxycineoles			
Ci 10	1	1.47	0.23
Ci 11	1	3.19	0.08
Hydroxy cineolic acids			
Ci 6	1	7.25	0.01
Ci 7	1	6.94	0.01
Ci 8	1	3.48	0.07
Ci 9	1	0.74	0.4
Ci 12	1	1.24	0.27
Ci 13	1	0.28	0.6
Ci 15	1	1.82	0.19
Ci 17	1	0.24	0.62
Ci 18	1	7.17	0.01
Ci 19	1	0.0003	0.99
Ci 20[1]	1	5.62	0.02

[1]Recovery of free Ci 20 was greater than its total recovery. This may be due to its chromatography deteriorating, since this minor metabolite was more polar than other metabolites (except Ci 19).

Further clarification of the extent of conjugation is presented in Table 10.5 for individual hydroxycineoles and cineolic acid metabolites, which accounted for the majority of conjugation, plus total metabolites. For Ci 2, 3, 4 and 5, the fraction excreted in the conjugated form increased significantly throughout the treatment. There was no detectable increase in the fraction of total metabolites conjugated.

Urinary glucuronic acid measurements for each day are shown in Figure 10.7. There was a reasonable correlation between dietary 1,8-cineole concentration and urinary glucuronic acid excretion ($df = 17$, $R^2 = 0.68$, $P < 0.001$). Values for individual possums are reported in Appendix 8 - Table A8.2.

There was also reasonable correlation between the conjugated metabolites excreted and the urinary glucuronic acid concentration ($df = 17$, $R^2 = 0.68$, $P < 0.001$).

Table 10.5. Fraction of metabolites excreted as hydrolysable conjugates for the two metabolite groups that underwent the majority of conjugation plus the sum of all metabolites in Experiment 1 (mean ± sd, n = 6).

	Day 3	Day 9	Day 17	Statistical comparison[1]	
	Fraction of metabolites conjugated			F	*P* -value
Hydroxy cineoles					
Ci 1	0.82 ± 0.28	0.88 ± 0.16	0.90 ± 0.15	0.26	0.77
Ci 3	0.47 ± 0.20	0.64 ± 0.19	0.91 ± 0.10	10.14	0.002
Ci 5	0.41 ± 0.47	0.52 ± 0.30	0.96 ± 0.08	4.52	0.03
Cineolic acids					
Ci 2	0.36 ± 0.22	0.38 ± 0.24	0.79 ± 0.16	7.81	0.005
Ci 4	0.09 ± 0.14	0.09 ± 0.21	0.54 ± 0.24	9.86	0.002
Sum all metabolites	0.28 ± 0.15	0.3 ± 0.14	0.41 ± 0.23	0.95	0.41

[1]Anova (single factor) comparing the fraction of conjugated metabolites between the three sample days for each metabolite (*df* = 2).

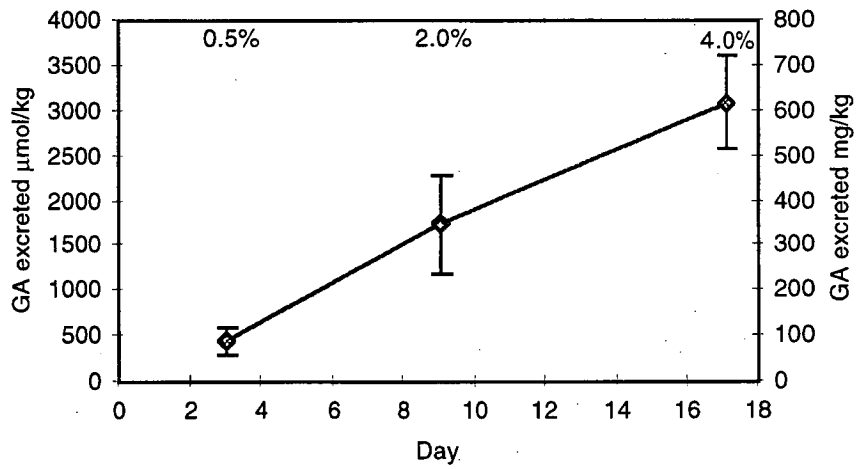


Figure 10.7. Urinary glucuronic (GA) acid excretion (µmol/kg and mg/kg; mean ± se, n = 6) by possums increased significantly as 1,8-cineole intake increased (Anova (single factor) *df* = 2, F = 8.94, *P* = 0.003) in Experiment 1.

10.2.3. Discussion

The aim of this first experiment was to determine whether a threshold level of 1,8-cineole intake was evident over a concentration range that would exceed levels expected to be encountered in a natural eucalypt leaf diet. Individual possum data revealed that possums BP 7, 8, 11 and 12 did not increase their intake of 1,8-cineole

above critical levels, ranging between 3 and 4 g/kg daily. There were no conclusive results for BP 9 and 10, possibly suggesting they had higher thresholds.

As the dietary 1,8-cineole concentration increased, possums appeared more reluctant to eat the diet. At low concentrations possums awoke and ate some of the food as soon as it was offered. As the concentration increased, they showed little enthusiasm for eating. They also became messier with their eating, sifting through the food presumably looking for more palatable portions. Therefore, the diet became less palatable as the concentration increased. The taste or smell of 1,8-cineole may therefore contribute directly to reduction in food intake.

Another recent report of a similar feeding experiment (Lawler *et al.* 1999) found that possums ingested 3.5 $g/kg^{-0.75}$ of 1,8-cineole without any indication of a threshold intake being reached. The equivalent kg^{-1} value for ingested 1,8-cineole is considerably lower. Therefore it seems the experiment was discontinued before 1,8-cineole concentrations comparable to those in this experiment were reached and that were associated with 1,8-cineole thresholds.

There was no evidence of saturation of oxidative enzymes at the higher intakes since each metabolite group accounted for the same proportion of the total metabolites recovered (Figure 10.4). This is further supported by the excretion pattern of individual metabolites (Figure 10.3.). Although the amounts excreted changed each day, there was no major variation in the relative abundance of any metabolite.

Urinary glucuronic acid measurements suggest that, on a 1,8-cineole-free diet, only minor amounts of glucuronic acid were excreted. As 1,8-cineole intake increased a proportional increase in urinary glucuronic acid excretion resulted. This supports our assumption that hydrolysable metabolites were glucuronide conjugates. The correlation between glucuronic acid concentrations and conjugated metabolites is further evidence that glucuronidation accounts for the hydrolysable metabolite fraction.

Conjugation was important in the elimination of 1,8-cineole metabolites in brushtail possums. Approximately 30 - 40% of the recovered metabolites were excreted as glucuronides. Although not statistically significant, there was an overall increase in the fraction of glucuronidated metabolites, due to increases in Ci 2, 3, 4 and 5, throughout the experiment which possibly suggest glucuronyl transferase enzymes were induced. McLean *et al.* (1993) reported induction of these enzymes in ringtail possums over the first few days after replacing an artificial diet with *E. radiata*. The greatest increase in the fraction conjugated occurred between days 9 and 17.

The increased fraction of glucuronidated metabolites was the only apparent alteration in the pattern of metabolism with increasing intakes of 1,8-cineole. The increase in 1,8-cineole intakes combined with a greater fraction of conjugated metabolites increases the demand for glucuronic acid as a substrate. As much as 3 g per day of glucuronic acid was excreted at the higher intakes of 1,8-cineole (Figure 10.7). If glucuronic acid supply was a limiting factor in the glucuronidation of the less polar metabolites and these same metabolites were not readily excreted or further oxidised, then elevated blood levels could occur. This could potentially result in a negative feedback causing a reduction in food (and 1,8-cineole) intake.

A preliminary understanding of the metabolic pathways utilised in the metabolism of

1,8-cineole in the brushtail possum was possible, despite not having elucidated the structures of all metabolites. In total eighteen metabolites were quantified in the brushtail possum and many of these were present in both their free and conjugated form. This suggests that a multiplicity of metabolic pathways are utilised by the brushtail possum to metabolise 1,8-cineole.

Metabolites encompassed a range of oxidation outcomes, presumably resulting from activity from both the CYP and alcohol/aldehyde dehydrogenase enzyme systems. Oxidation by the microsomal CYP enzymes is responsible for the formation of hydroxycineole and dihydroxycineole metabolites in brushtail possums (G. Pass, personal communications). These metabolites then become substrates for the dehydrogenase enzymes to produce the carboxylic acid metabolites, cineolic and hydroxy cineolic acids. Interestingly, most of the metabolites excreted have been further oxidised to carboxylic acids. Hydroxycineole and dihydroxycineole metabolites accounted for less than 15 and 5% of the recovered dose, respectively. On the other hand, cineolic acids and hydroxy cineolic acids accounted for 25 - 30 % and 55 - 60 % of the recovered dose, respectively. Despite eleven hydroxy cineolic acids being detected, only two precursor dihydroxycineoles were found in measureable quanitites in possum urine. The cineolic acids were also found in greater amounts than their precursor hydroxycineole metabolites. Therefore, it would seem that the dehydrogenase enzymes are highly efficent at oxidising alcohol and hydroxy compounds to carboxylic acids and therefore have a critical role in the metabolism of 1,8-cineole in possums.

The ratio of 9- to 7-oxidised metabolites (approximately 4:1 and 10:1 for hydroxycineoles and cineolic acids, respectively) suggest that the C9 position is the most reactive site for oxidation. It may therefore be reasonable to assume that the major hydroxy cineolic acid metabolites are derivatives of 9-cineolic acid.

The acidity of the cineolic and hydroxy cineolic acid metabolites was reflected in the pH of the urine. A gradual but marked decrease from pH 8 to pH 6 was a result of acidic metabolites. The formation of acidic metabolites results in an acid load which could potentially upset acid-base homeostasis in the animals (Foley *et al.* 1995). On a 1,8-cineole free diet, urine pH was relatively high and presumably has the capacity to buffer a large acid metabolite load.

Recovery of estimated 1,8-cineole intake ranged from 0.40 ± 0.16 to 0.55 ± 0.21 (mean ± sd) on the urine sample days (Table 10.3). Since this fraction was consistent between the urine sample days, the most likely explanation for this relatively low recovery was the estimate of 1,8-cineole intake. The intake was calculated from the weight of food consumed multiplied by the 1,8-cineole concentration (wet weight). Overestimation of food, and therefore 1,8-cineole intake, was likely. Although 1,8-cineole is not highly volatile (bp 176-177°C) an unknown percentage of the dose would be expected to evaporate depending on degree and frequency of disturbance of the food and surface area of exposed food. Furthermore, it was often difficult to retrieve all food wastes for inclusion in the weight of refused food, particularly if a possum had been very messy. Evaporative losses of water were also subject to the same inter-possum variations. The weight of food intake was adjusted by the evaporative loss measured in a control food tray. This was a poor estimate of evaporative loss as the food was allowed to sit undisturbed, minimising the evaporative surface. Measuring the dry weight of food intake would have overcome

some of the vaiability and have resulted in a more accurate measurement of intake.

Initially, administering 1,8-cineole in the form of gelatin-acacia microcapsules was considered. Microencapsulation of semi-volatile or lipophilic compounds has many applications, including as a relatively novel dosage form in the pharmaceutical industry (Jizomoto *et al.* 1993), as well as incorporating feeding deterrents into insect diets (Gunasena *et al.* 1988; Usher *et al.* 1989; Clancy *et al.* 1992). The main advantage for this study would have been to protect 1,8-cineole from evaporation. However, other researchers (I.R. Lawler and W.J. Foley, James Cook University, Townsville, Queensland) attempting to use microencapsulated terpenes as a more realistic way of packaging dietary terpenes, recommended caution. Recently Lawler and Foley (1999) summarised some of the problems encountered with this technique. The diet had to be substantially modified to allow the incorporation of capsules. Furthermore, ringtail possums stopped eating for a number of days after this diet was offered for a single night. These considerations combined with the prohibitive cost of manufacturing, outweighed the potential advantages of investigating this technique further.

The shortcomings described above had minimal effect on the metabolism studies apart from resulting in a poor estimation of 1,8-cineole intake which consequently affected 1,8-cineole recovery as well as increasing variability (or standard deviations) of both observational and metabolism study results.

Other routes of elimination may be responible for some of the unaccounted dose of 1,8-cineole. Biliary excretion of metabolites was also investigated in Experiment 2. In summary, however, metabolites were found in faecal extracts but accounted for a only very small amount of the total ingested dose. No unchanged 1,8-cineole was detected in urine or faecal extracts which was in agreement with Southwell *et al.* (1980) who reported on steam distillation of urine and faecal extracts from 1,8-cineole fed possum. Pharmacokintetic studies of inhaled terpenes in humans report that about 8 % of inhaled α-pinene is eliminated unchanged in expired air (Falk *et al.* 1990; Levin *et al.* 1992). This was also found to be the case in brushtail possums and was the basis of a novel approach to studying the pharmacokinetics of 1,8-cineole in possums reported in Appendix 1.

Delayed absorption or elimination of 1,8-cineole may also account for low recoveries. Post 1,8-cineole treatment washout was not examined in this experiment but was given special consideration in Experiment 2.

The amounts of 1,8-cineole ingested throughout the experiment, especially at the higher concentrations, were substantial. On day 17, possums were consuming an estimated average of 12 g of 1,8-cineole per day. Given the relatively small size of brushtail possums (mean weight 3.6 kg), this amount of 1,8-cineole represents a major detoxification challenge for possums, even if the dose is somewhat overestimated. From the insight into the detoxification processes we have acquired in this experiment it appears the possums have a very high capacity to cope with large amounts of 1,8-cineole in their diet.

A critical daily intake threshold of 1,8-cineole was clearly demonstrated in four out of six possums. This threshold was achieved during a gradual increase in dietary concentration of 1,8-cineole. Although the oxidation pathways showed no sign of

saturation across the concentration range, the role of conjugation of lesser oxidised metabolites became increasingly more important as the dose increased. Experiment 2 was designed to address the following specific questions arising from Experiment 1:

1) If naive possums are suddenly challenged with a diet high in 1,8-cineole (eg. 4 % wet weight) is there an adjustment period in their food intake?

2) When naive possums are suddenly challenged with a diet high in 1,8-cineole, is there any metabolic evidence to indicate induction of oxidation enzymes or glucuronyl transferases?

3) What is the washout period of metabolites after the cessation of a diet high in 1,8-cineole in possums?

10.3. Experiment 2 - Induction of oxidative enzymes

The second experiment was designed to address the questions raised in the last section. Naive possums were challenged with the maximum concentration of 1,8-cineole used in Experiment 1 (4 % wet weight) for 5 days. Observational data were collected before, during and after the 1,8-cineole treatment and the data correlated to the pattern of metabolism. If induction of 1,8-cineole detoxifying enzymes occurs, we would expect to see a delay in possums reaching the same level of 1,8-cineole consumption observed in Experiment 1. Furthermore, by examining urinary metabolites over the first few days of 1,8-cineole ingestion, alteration in the pattern of excretion of metabolites may become apparent.

10.3.1. Methods

Possums were again maintained in metabolism cages on a basal diet for two days (days 1 and 2). On the third day they were challenged with a diet containing 4% wet weight of 1,8-cineole. This treatment was continued for 5 days. The 1,8-cineole free diet was reintroduced for a further 2 days to observe recovery and metabolite washout. Techniques used for preparing diets and collecting samples were the same as those described for Experiment 1 - Section 1.2.1.

Urine analyses were the same as those described in Chapter 9 and used for Experiment 1. Detailed analysis of faecal extracts are also reported for this experiment using the method described in Chapter 9 - section 9.2.6.1.

10.3.2. Results

10.3.2.1. Dietary intake

Observational results for food and 1,8-cineole intake are reported in Figure 10.8. Food intake was reduced significantly whilst possums were being fed the 4 % diet and increased again upon cessation of 1,8-cineole. Food and 1,8-cineole intake tended to increase throughout the 1,8-cineole treatment, although this was not statistically

significant.

Urine output decreased with the commencement of 1,8-cineole treatment on day 3 and began to increase again towards the end of the treatment period (Figure 10.9-A). Reduced urine production probably directly reflects food intake. Figure 10.9-B shows a marked drop in urine pH with 1,8-cineole treatment.

Faecal excretion was severely affected by the 1,8-cineole treatment (Figure 10.9-C). In fact output was restricted so severely that on each treatment day up to three possums failed to defecate. All possums defecated on days 1 and 2, although weights were not recorded. This observation was consistent with Experiment 1 where although faecal output was variable, there was a consistent reduction in output as 1,8-cineole concentration increased.

10.3.2.2. Metabolite analyses

Urine samples from days 3, 4, 5, 7, 8 and 9 were analysed for urinary metabolites and glucuronic acid. 1,8-Cineole intakes for these days as well as the treatment regime of 1,8-cineole are summarised in Table 10.6. Individual possum data, including metabolite analyses, are reported in Appendix 8 - Table A8.3.

The same eighteen metabolites were identified and quantified as those reported in Experiment 1. Recoveries for individual metabolites (μmol/kg) are reported graphically in Figure 10.10 for each day urine samples were analysed. Although the y-axis values changed for each day, the relative abundance of each metabolite is easily visualised. There were some marked changes in the pattern of excretion throughout the experiment. On day 3, the first 1,8-cineole treatment day, the least oxidised metabolite, Ci 3 (9-hydroxycineole), dominated the metabolite profile and there were only small amounts of the more oxidised metabolites. Throughout days 4, 5 and 7 the proportion of more oxidised metabolites increased substantially. A transition occurred from Ci 3 to Ci 2 as the most abundant metabolite . On the first washout day (day 8), all metabolites diminished proportionally except Ci 19 and 20. By the second washout day, the least oxidised metabolites again dominated the metabolite profile.

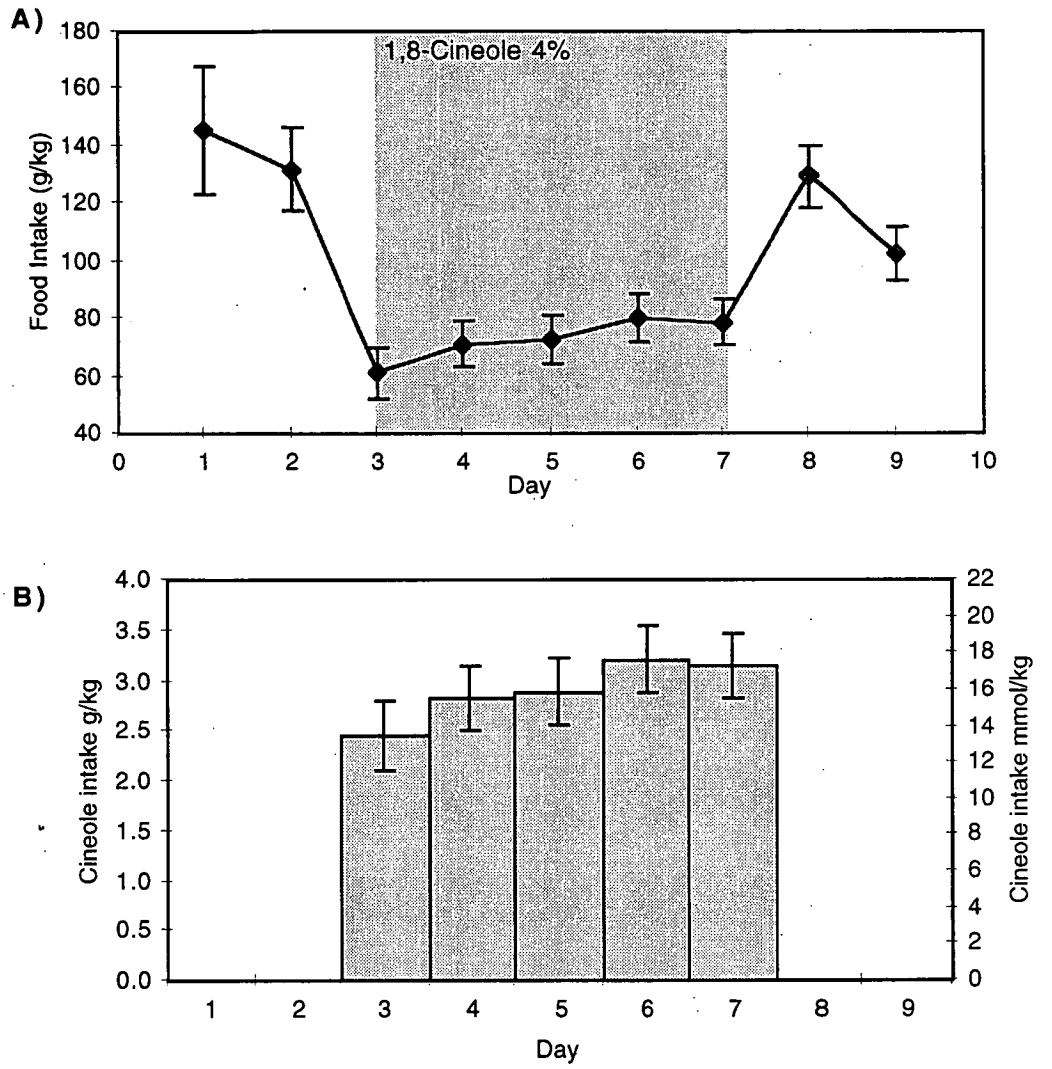


Figure 10.8. Food and 1,8-cineole intake. A) Food intake (g/kg; mean ± se) decreased with the introduction of the 1,8-cineole diet (Anova (single factor) *df* = 8, F = 7.03, *P* < 0.001). The 1,8-cineole treatment period is indicated by shading. B) 1,8-Cineole intake (g/kg and mmol/kg) tended to increase throughout the treatment period. All data shown as mean ± se (n = 6).

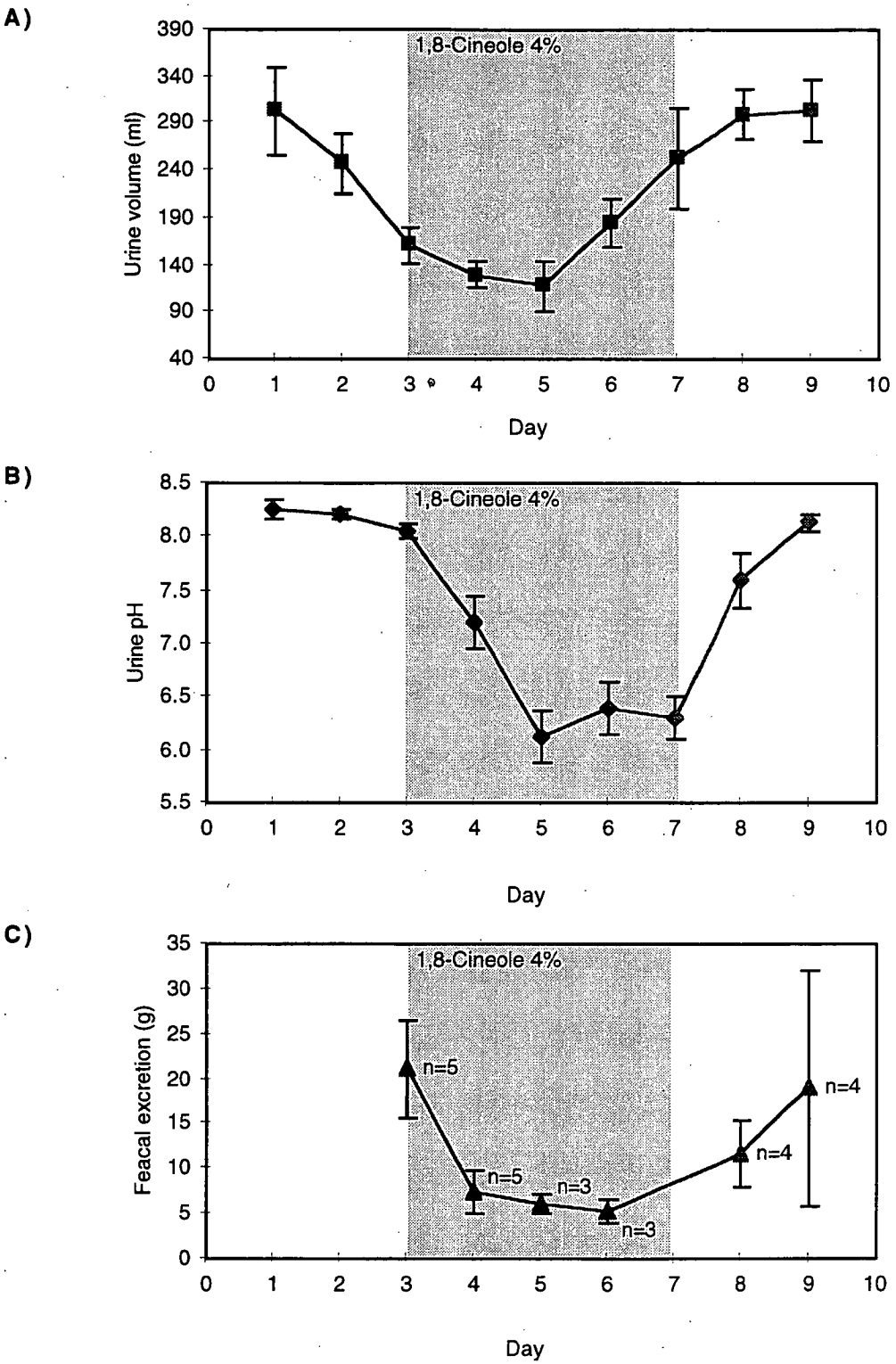


Figure 10.9. Urine and faecal excretion. A) Urinary output (ml) decreased significantly during the 1,8-cineole treatment (df = 8, F = 5.25, P < 0.001), B) urine pH decreased significantly during the treatment (df = 8, F = 23.03, P < 0.001) and C) faecal output (g) was only measured for days 3 to 9. However, fewer possums produced faecal pellets and faecal weights were reduced during the treatment period. Values reported as mean ± se for n = 6 possums and Anova (single factor) was used for statistical comparisons. The 1,8-cineole treatment period is indicated by shading.

Table 10.6. Dietary 1,8-cineole concentrations (%) and 1,8-cineole intakes for possums (n = 6) on urine sample days of Experiment 2.

	Day 3		Day 4		Day 5	
	Mean	sd	Mean	sd	Mean	sd
1,8-Cineole concentration						
% wet weight	4.0		4.0		4.0	
1,8-Cineole intake						
g	8.8	3.7	10.1	3.5	10.1	3.0
mmol	57.0	24.1	65.8	23.0	65.8	19.7
mmol/kg	15.9	5.6	18.4	5.1	18.8	5.4

	Day 7		Day 8		Day 9	
	Mean	sd	Mean	sd	Mean	sd
	4.0		0.0		0.0	
	10.9	2.3	0	0	0	0
	71.0	14.8	0	0	0	0
	20.4	5.3	0	0	0	0

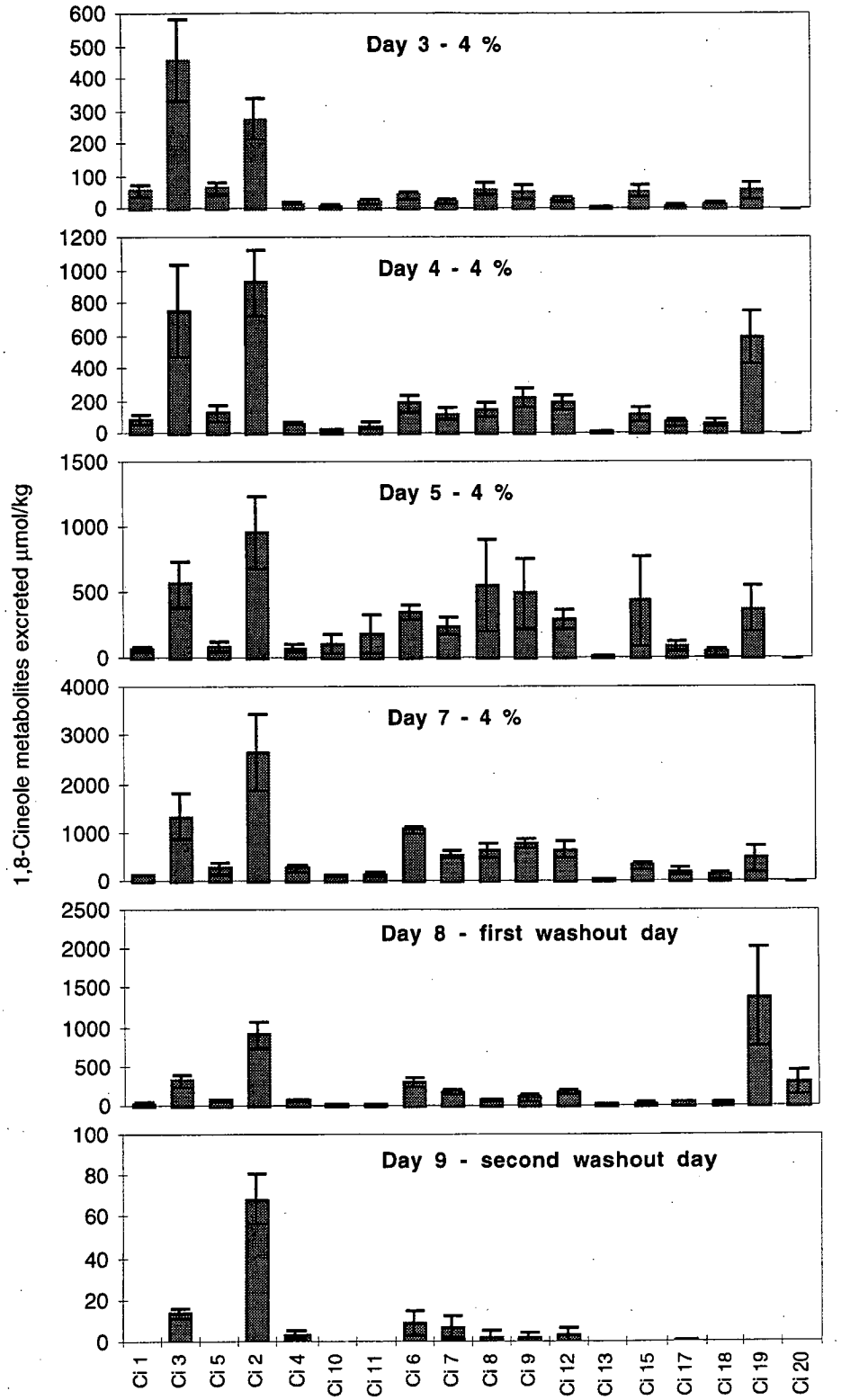


Figure 10.10. Individual 1,8-cineole metabolite recoveries (µmol/kg), after hydrolysis, for each urine samples day. Metabolites are identified in Chapter 9 - Figure 9.6 and Table 9.3 and are reported as mean ± se (n = 6).

The recovery of 1,8-cineole as both free and total metabolites is reported in Table 10.7. Although the daily intake of 1,8-cineole did not vary significantly, the fractional daily recovery of total metabolites started very low and increased greatly throughout the treatment period. As expected, the fractional recovery of total metabolites was significantly greater than the recovery of free metabolites on each day of the treatment (Table 10.7).

Table 10.7. Fractional recovery of 1,8-cineole intakes as urinary metabolites (n = 6).

	Fraction of administered dose recovered							
	Day 3		Day 4		Day 5		Day 7	
Possum	Free	Total	Free	Total	Free	Total	Free	Total
Mean	0.05	0.09	0.12	0.20	0.09	0.24	0.24	0.46
sd	0.05	0.06	0.05	0.13	0.05	0.19	0.10	0.10

Hydrolysis caused an increase in metabolite recovery each day (Anova (two factor with replication) $df = 1, F = 17.52$ $P < 0.001$).

The recovery of hydrolysed metabolites increased significantly throughout the treatment period (Anova (single factor) $df = 3, F = 8.90$, $P < 0.001$).

As in Experiment 1, the overall pattern of excretion of metabolite groups was examined as the percentage of recovered 1,8-cineole. Figure 10.11 shows a transition in the pattern of metabolite excretion during the first three days of the treatment which was discussed earlier in this section. On the first day, the hydroxycineoles accounted for about 48 % of recovered 1,8-cineole but this dropped to about 15 % and the percentage of cineolic acids and hydroxy cineolic acids increased correspondingly. Once stabilised, the percentages of each group were comparable to those reported in Experiment 1.

A similar pattern of metabolite excretion continued into day 8, the first washout day. The percentage of hydroxy cineolic acid dropped as the percent of cineolic acid rose. In contrast, at the beginning of the treatment the hydroxycineoles accounted for the greatest percentage of metabolites.

Figure 10.12 reports the molar recoveries of free and total metabolites for each group. Only total metabolites were measured during the washout days (days 8 and 9). The difference between total and free levels represents hydrolysable conjugation. Hydrolysis resulted in significant increases in the recovery of total metabolites, hydroxycineoles, cineolic acids and dihydroxycineoles (Figure 10.12). Recovery of hydroxy cineolic acids did not increase with hydrolysis.

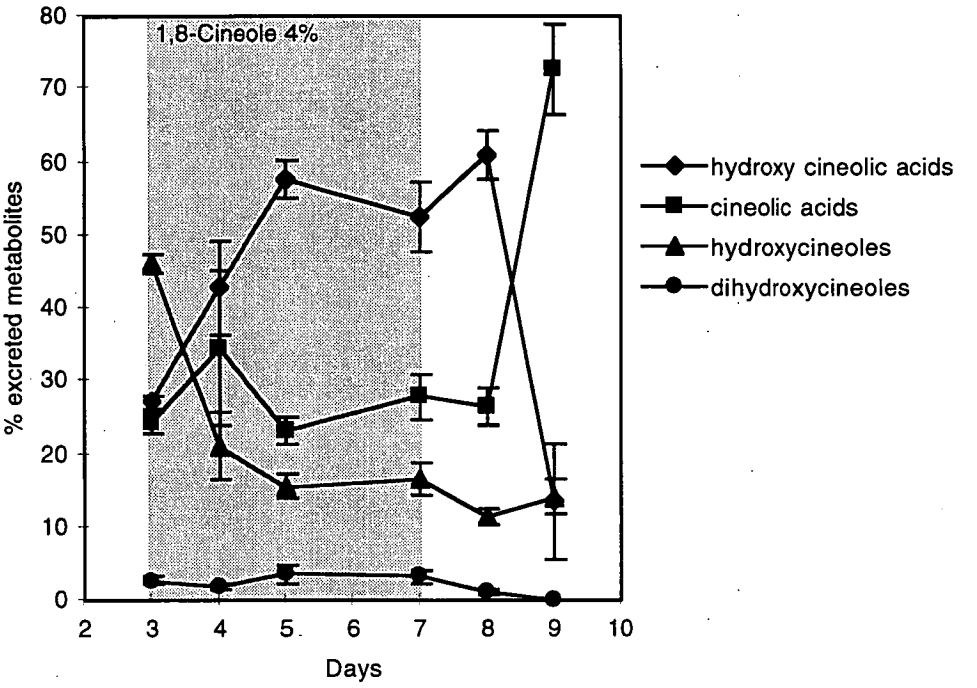


Figure 10.11. Recovery of 1,8-cineole metabolite groups as a percentage of total metabolite recovery for brushtail possums in Experiment 2 (mean ± se, n = 6). The 1,8-cineole treatment period is indicated by shading.

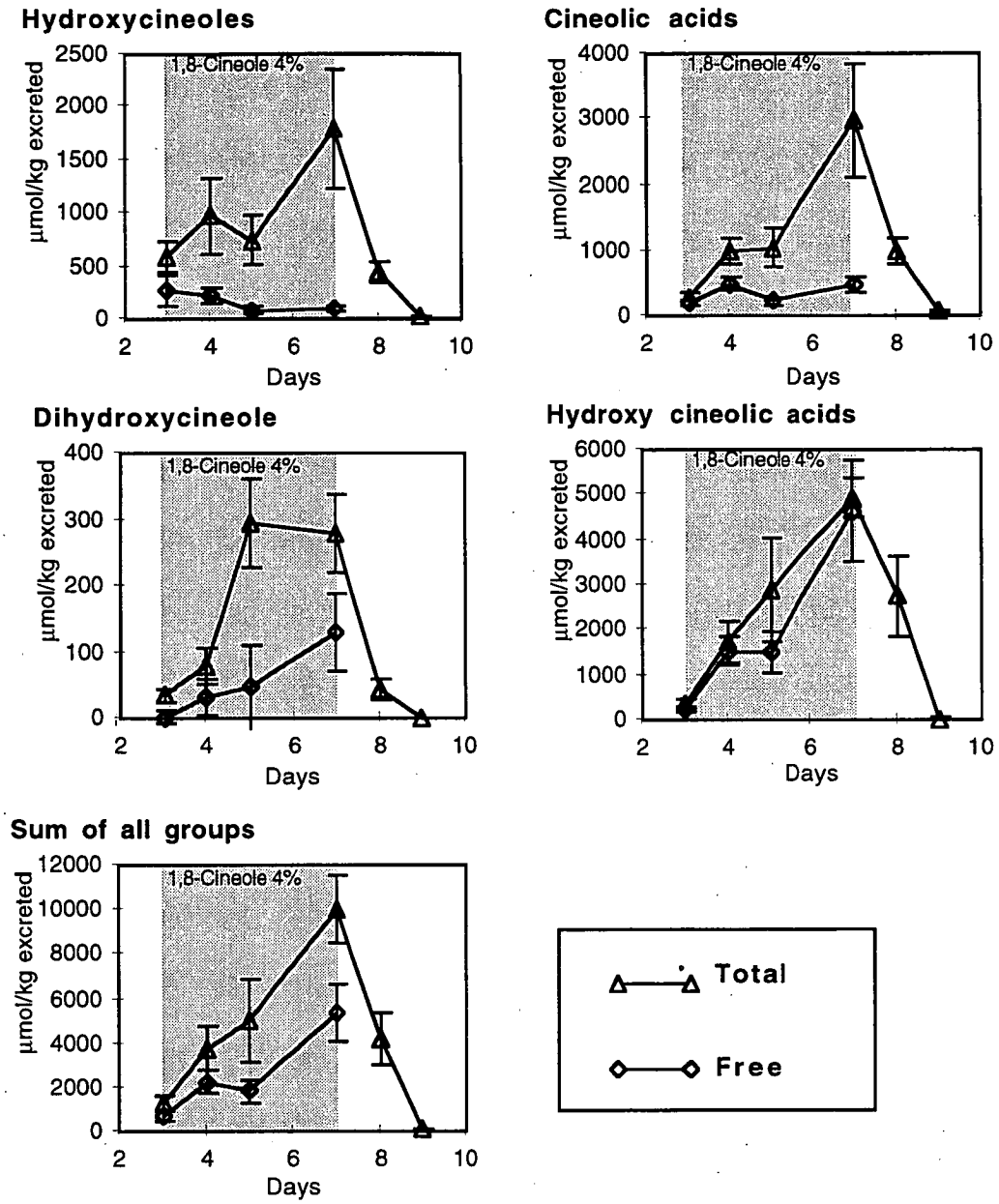
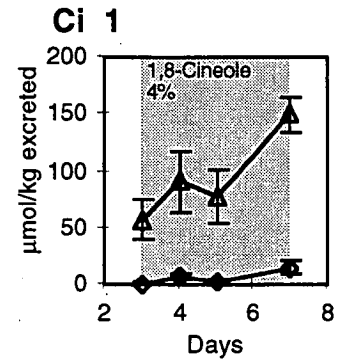
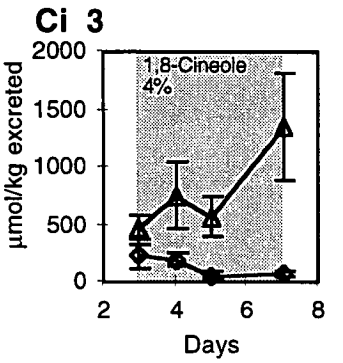


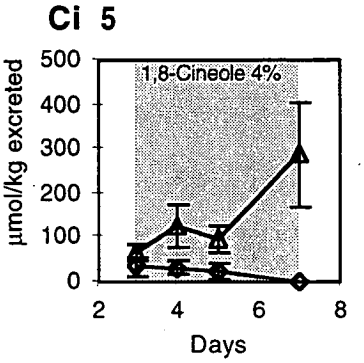
Figure 10.12. Comparison of free and total metabolites for each metabolite group for Experiment 2. Values are µmol/kg (mean ± se, n = 6). The 1,8-cineole treatment period is indicated by shading. Hydrolysis increased the recovery of hydroxycineoles, cineolic acids, dihydroxycineoles and total metabolite recovery (Anova (two factor with replication) df = 1, F = 20.88, P < 0.001; df = 1, F = 16.78, P < 0.001; df = 1, F = 4.20, P = 0.05 and df = 1, F = 4.08, P = 0.002, respectively). Recovery of hydroxy cineolic acids did not increase with hydrolysis (Anova (two factor with replication) df = 1, F = 1.26, P = 0.27).

Free and total molar recoveries for individual metabolites during the 1,8-cineole treatment period are shown in Figure 10.13 and the associated statistical comparisons between free and total levels are reported in Table 10.8. The conjugation pattern of individual metabolites belonging to the hydroxycineole, cineolic acid and dihydroxycineole groups reflected the overall conjugation of their respective groups. The least oxidised metabolites, the hydroxycineoles, underwent the greatest degree of conjugation.

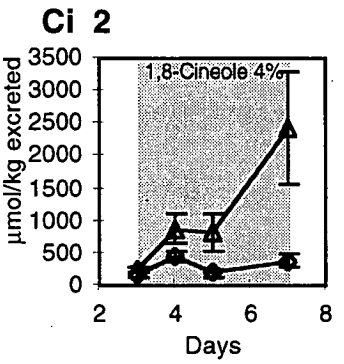
1) Hydroxycineoles

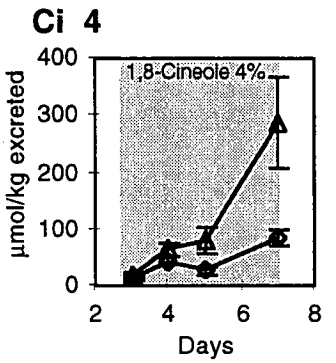




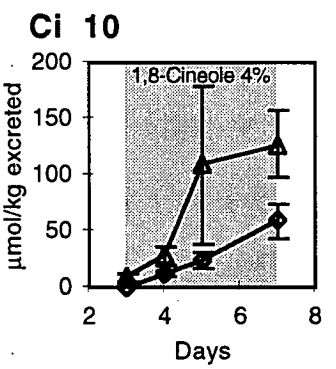


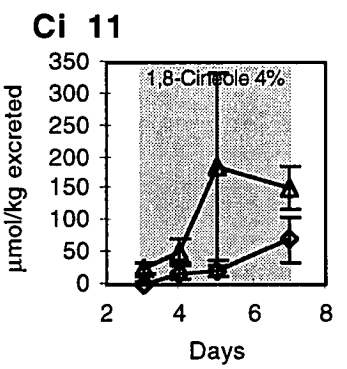
2) Cineolic acids



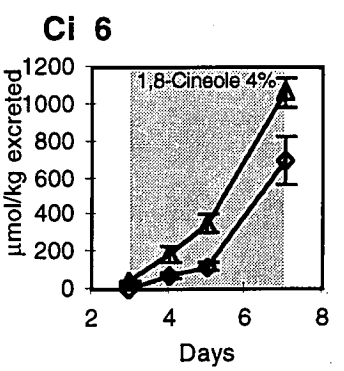


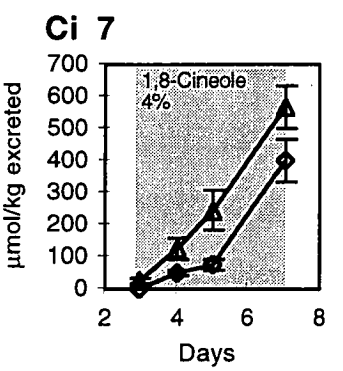
3) Dihydroxycineoles

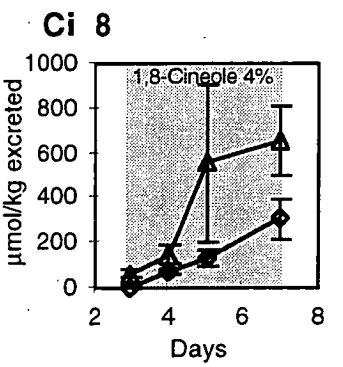




4) Hydroxy cineolic acids







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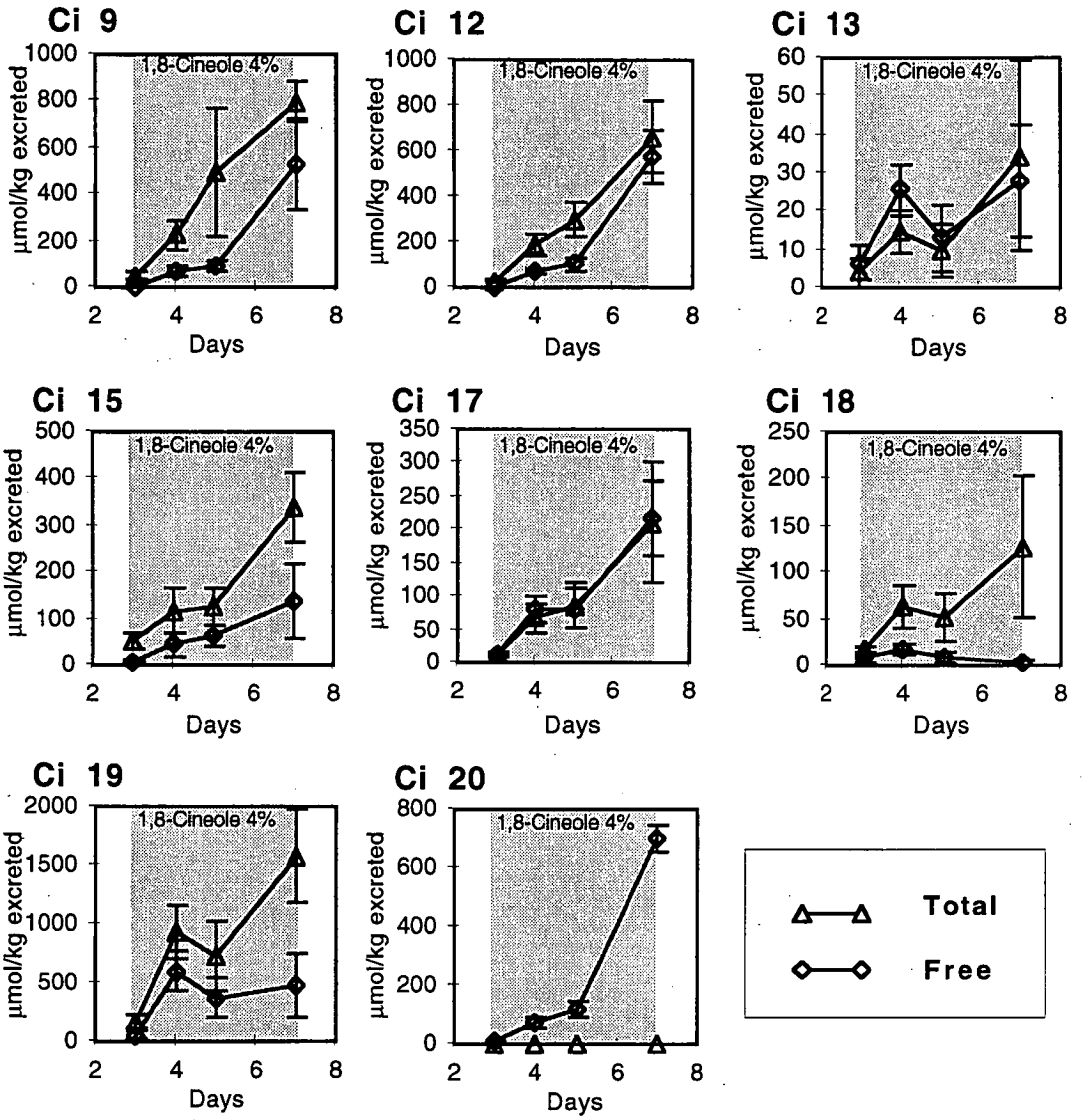


Figure 10.13. Comparison of free and total metabolite recoveries for individual metabolites in Experiment 2. Values are µmol/kg (mean ± se, n = 6). Anova comparisons of free and total recoveries for each metabolite are reported in Table 10.8.

Table 10.8. Statistical comparisons of total and free metabolites for the individual metabolites shown in Figure 10.13.

Metabolite	Anova two factor with replication (n = 6)		
	df	F value	*P* value
Hydroxy cineoles			
Ci 1	1	60.9	<0.001
Ci 3	1	183.32	<0.001
Ci 5	1	12.78	<0.001
Cineolic acids			
Ci 2	1	17.34	<0.001
Ci 4	1	10.84	0.002
Dihydroxycineoles			
Ci 10	1	5.19	0.03
Ci 11	1	3.61	0.06
Hydroxy cineolic acids			
Ci 6	1	5.96	0.02
Ci 7	1	12.95	<0.001
Ci 8	1	5.15	0.03
Ci 9	1	5.96	0.02
Ci 12	1	3.84	0.06
Ci 13	1	0.1	0.75
Ci 15	1	8.12	0.007
Ci 17	1	0.02	0.89
Ci 18	1	6.92	0.01
Ci 19	1	8.51	0.006
Ci 20[1]	1	37.01	<0.001

[1]Recovery of free Ci 20 was greater than its total recovery. See Table 10.4 for explanation.

In this experiment, seven of the hydroxy cineolic acid metabolites were found to have undergone significant hydrolysable conjugation, although conjugation was not as extensive as the less oxidised metabolites. As mentioned in Experiment 1, the anomalous result of Ci 20, in which no recovery of total metabolite was detected, was due to its poor chromatography.

The extent and pattern of conjugation was further clarified by considering the proportion of metabolites excreted as conjugates throughout the sample days. Table 10.9 reports the fraction of all metabolites conjugated as well as individual hydroxycineoles and cineolic acids since they underwent the most extensive conjugation. For each metabolite in these two groups, except Ci 1, the fraction excreted in the conjugated form increased throughout the treatment. The increase in the fraction conjugated of these metabolites was not reflected in the fraction of total metabolites conjugated.

Table 10.9. Fraction of metabolites excreted as hydrolysable conjugates for the two metabolite groups that underwent the majority of conjugation (hydroxycineoles and cineolic acids) plus all metabolites grouped together, in Experiment 2. Results are reported as mean (n = 6).

	Day 3	Day 4	Day 5	Day 7	Statistical comparison[1]	
	Fraction of metabolites conjugated (mean)				F	*p* -value
Hydroxy cineoles						
Ci 1	0.99	0.76	0.95	0.86	1.32	0.30
Ci 3	0.54	0.56	0.87	0.93	3.37	0.04
Ci 5	0.52	0.55	0.73	1.00	0.12	0.12
Cineolic acids						
Ci 2	0.34	0.49	0.74	0.85	6.60	<0.001
Ci 4	0.18	0.29	0.61	0.68	9.69	<0.001
Sum all	0.48	0.34	0.60	0.49	1.54	0.24

[1]Anova (single factor) comparing the fraction of conjugated metabolites between the four sample days for each metabolite (*df* = 3).

Urinary glucuronic acid was measured directly and the excretion of glucuronic acid increased throughout the treatment (Figure 10.14). There was a good correlation between 1,8-cineole intake and urinary glucuronic acid excretion ($df = 23$, $R^2 = 0.71$, $P < 0.001$). Values for glucuronic acid and conjugated metabolites are recorded for individual possums in Appendix 8 - Table A8.4.

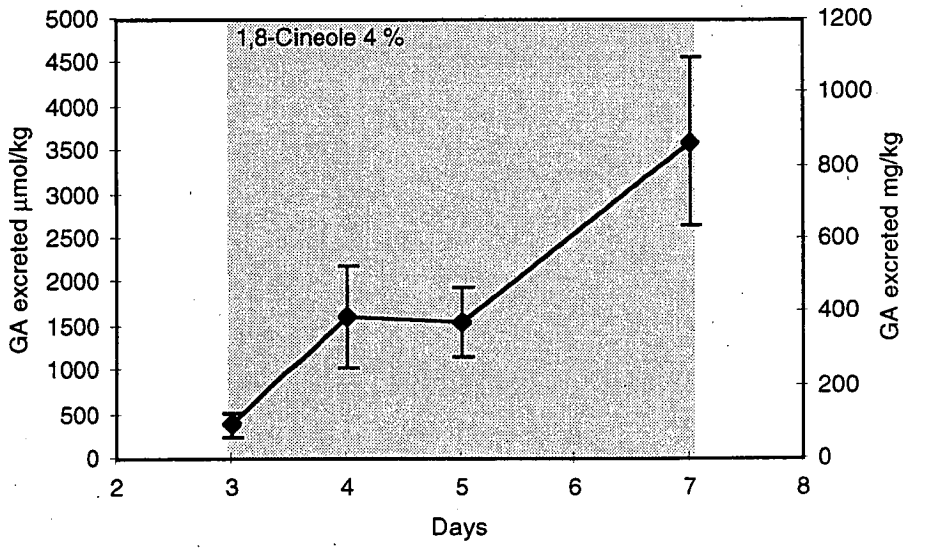


Figure 10.14. Urinary glucuronic (GA) acid excretion (µmol/kg and mg/kg; mean ± se, n = 6) by possums in Experiment 2 increased throughout the 1,8-cineole treatment period (Anova (single factor) *df* = 3, F = 4.93, *P* = 0.01).

Faecal pellets collected in Experiment 2 were analysed for unchanged 1,8-cineole or its metabolites. A sample of a gas chromatogram of a faecal extract is shown in Chapter 9 - Figure 9.11. Figure 10.15. shows the range and abundance of metabolites quantified in the faeces for both unhydrolysed and hydrolysed extracts from day 6 of the experiment. Visual comparison of the metabolite patterns in faecal and urine extracts (Figure 10.10 compared to Figure 10.15) shows that the less oxidised metabolites are under represented. This may be due to the molecular weights of these glucuronides (MW = 346 for hydroxycineole glucuronides) being slightly lower than those more extensively excreted by biliary excretion (MW = 376 for hydroxy acid cineole glucuronides). Conjugated metabolites excreted by biliary excretion appear to be hydrolysed during their passage through the gastrointestinal tract as there was no significant difference in free and total metabolite recovery.

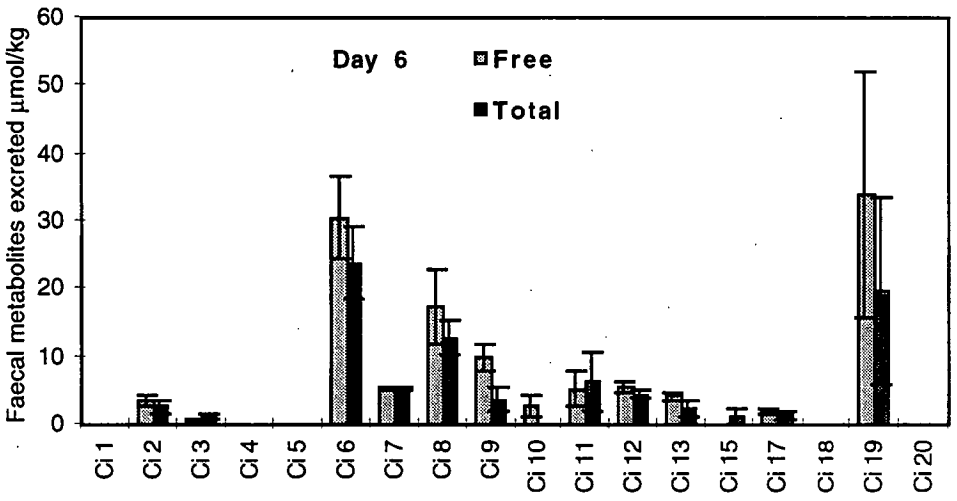


Figure 10.15. Free and total recoveries (µmol/kg) of each 1,8-cineole metabolite in possum faecal extracts from day 6 of Experiment 2. Metabolites are identified in Chapter 9 - Figure 9.6 and Table 9.3 and are reported as mean ± se, n = 3.

Overall faecal metabolite output is shown in Figure 10.16. Faecal excretion of metabolites was delayed and was also prolonged beyond cessation of the 1,8-cineole treatment. It was not possible to determine the overall fraction of the ingested 1,8-cineole excreted as faecal metabolites as they were still being excreted at the end of the experiment. However, from the molar values of metabolites recovered it is evident that only a small portion (< 1 %) is excreted by this route.

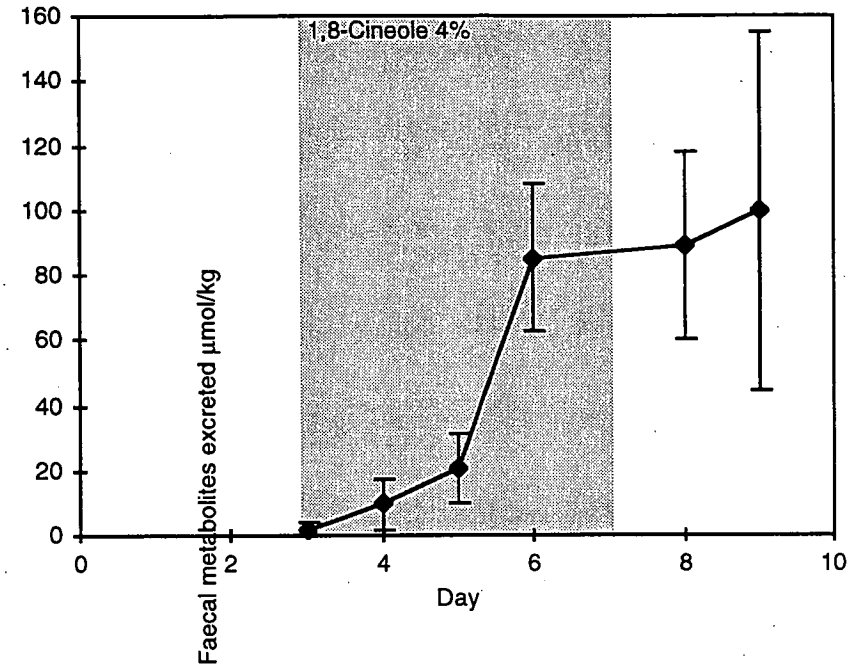


Figure 10.16. Total molar recovery of faecal metabolites (µmol/kg) for each day of analysis in Experiment 2. Values are reported as mean ± se and the 1,8-cineole treatment period is indicated by shading. See Figure 10.9-C for numbers of possums defecating on each day.

10.3.3. Discussion

The questions specifically addressed in this experiment are stated below and the outcomes discussed.

1) *If naive possums are suddenly challenged with a diet high in 1,8-cineole (eg. 4 % wet weight) is there an adjustment period in their food intake?*

Day 3, the first day of the 1,8-cineole treatment, resulted in the lowest food intake of the experiment. As the treatment continued, food intake tended to increase, although the increase was not statistically significant (Figure 10.8-A). Therefore there was no overtly significant adjustment period in food intake during the 5 days of a 1,8-cineole treatment. The trend to increased food intake translated into a modest, but again insignificant increase in 1,8-cineole intake (Figure 10.8-B).

The lower food intakes observed earlier in the study may reflect carryover from excessive food intake on pre-treatment days.

Comparing food and 1,8-cineole intake with Experiment 1 (Figures 10.1 and 10.8), intakes were slightly higher at 4% cineole in Experiment 1, although this was not statistically significant. Further comparisons between all experiments will be made at the end of this chapter.

2) *When naive possums are challenged with a diet high in 1,8-cineole, is there any metabolic evidence to indicate induction of oxidation enzymes or glucuronyl transferases?*

The day by day study of urinary metabolites provided evidence of induction of oxidative enzymes. On the first day of treatment the least oxidised metabolites, the hydroxycineoles, accounted for almost 50 % of the metabolites of which 9-hydroxycineole (Ci 3) was the major metabolite. On the second day, 9-cineolic acid (Ci 2) was the major metabolite while the hydroxy cineolic acids accounted for approximately 45 % of the metabolites. By the third day, 9-cineolic acid was still the most abundant metabolite, but the hydroxy cineolic acids combined accounted for 60 % of the metabolites.

The transition from lesser to more oxidised metabolites indicates induction of oxidative enzyme activity. It appears naive possums have a baseline capacity to produce oxygenated metabolites, however, induction of enzymes responsible for more extensively oxidised metabolites occurs over the first few days of exposure. This supports the presumption that different oxidative enzymes are utilised in stepwise oxidative reactions. It is suggested that cytosolic alcohol and aldehyde dehydrogenases catalyse the formation of carboxylic acid metabolites; if this is the case, then it is these enzymes that were being induced.

Induction of oxidation pathways would not have been apparent in Experiment 1 as the initial intake of 1,8-cineole was low compared to Experiment 2. Furthermore, the first urine sample to be analysed was from the second day of 0.5 % 1,8-cineole treatment. The results from Experiment 2 showed that the changes in excretion pattern of oxidised metabolites mostly occurred within the first day of exposure to 1,8-cineole (Figure 10.11).

The pattern of oxidation was similar between the two experiments once induction was established.

There was also evidence that glucuronidation was induced during the 1,8-cineole treatment. Table 10.9 showed that the conjugated glucuronide fraction increased across the treatment period for metabolites Ci 2, 3, 4 and 5 (although the values for Ci 5 were not statistically significant) in a similar manner as Experiment 1. It is interesting to note that on the final day of 1,8-cineole the fractions of Ci 1, 2, 3, 4 and 5 conjugated were comparable to the fraction conjugated on day 17 in Experiment 1.

3) *What is the washout period of metabolites after the cessation of a diet high in 1,8-cineole in possums?*

The washout of urinary metabolites after cessation of 1,8-cineole was clearly demonstrated in Figure 10.12. In the first 24 hours after 1,8-cineole cessation, day 8, significant amounts of each metabolite group were measured in the urine. Forty eight hours after 1,8-cineole was discontinued, metabolite levels had diminished to insignificant levels.

Despite the size of the ingested 1,8-cineole dose, possums were capable of eliminating it rapidly. The lipophilic nature of 1,8-cineole would suggest it fits a multi compartment pharmacokinetic model. If this is the case, then a relatively long terminal phase elimination half life would be expected due to a large volume of distribution. This has been demonstrated in humans after exposure to α-pinene by inhalation (Falk *et al.* 1990). If this was the case in brushtail possums, absorption of

all gut 1,8-cineole, metabolism and excretion was essentially complete within 48 h of discontinuing the diet. Preliminary studies on the pharmacokinetics of 1,8-cineole in brushtail possums are reported in Appendix 1.

Almost all the 1,8-cineole metabolites detected in urine extracts were also detected in the faecal extracts, although some were so minor they were not quantified (Figure 10.15). Few reports consider biliary excretion of conjugated metabolites as a route of elimination of terpenes in marsupial eucalypt folivores. Examination of faeces has been limited to searching for unchanged oil (Southwell *et al.* 1980; Foley *et al.* 1987). However, a study examining the fate of *l*-menthol in rats reported extensive enterohepatic circulation of menthol glucuronide and as much as half of the dose was recovered from the faeces (Yamaguchi *et al.* 1994). Another study also reported on the conjugated biliary metabolites of the terpene pulegone (Thomassen *et al.* 1991). It was therefore not surprising to detect metabolites in faecal extracts from possums, especially considering the large amount of 1,8-cineole being ingested. However, that essentially all the urinary metabolites were detected in the faeces, although in minor amounts in some instances, was surprising. The absolute molar amount of faecal metabolites were very small and accounted for only a small portion of the 1,8-cineole ingested.

There was a marked delay of two or three days in the faecal excretion of metabolites and clearance of metabolites by this route continued well after 1,8-cineole was discontinued. The highest concentration of metabolites detected occurred on the final day of sampling (day 9). Further washout days would be required to determine how long metabolites were excreted in the faeces. The lag in excretion is not surprising given the reduction in faecal output observed at the onset of 1,8-cineole treatment (Figure 10.9-C).

Biliary excretion of metabolites would require metabolites to be conjugated, as only larger molecular weight compounds (greater than 300 - 500) are excreted by this route (Pratt and Taylor 1990). Yet, metabolites were excreted in their free form as hydrolysis did not increase recovery from faeces. Therefore hydrolysis of metabolites occurred *in vivo*, presumably allowing the reabsorption and reuse of glucuronic acid. Enterohepatic circulation of 1,8-cineole metabolites is also possible and may have contributed to the lag in metabolite excretion.

The lower molecular weight metabolites were under-represented in the faecal metabolite profile compared to urine. Hydroxycineole glucuronides and cineolic acid glucuronides have molecular weights of 346 and 360 respectively. That these metabolites do not undergo biliary excretion confirms that molecular weights of 1,8-cineole glucuronides are borderline for biliary excretion (molecular weights of hydroxy cineolic acid glucuronides are 376)

Factors contributing to the relatively poor recoveries of ingested 1,8-cineole are considered in detail in Experiment 1. Metabolite recovery in the washout days were not incorporated into the total recovery of 1,8-cineole doses, and therefore would increase the overall recovery significantly.

A suprising finding of Experiment 2 was that the fraction of 1,8-cineole recovered increased significantly throughout the treatment period. After the first day of treatment the recovery was very low (0.09). A possible explanation is that the 1,8-

cineole in the food may not have been completely absorbed and therefore that days intake carrying over to the following days excretion. The recovery increased to 0.46 on last day of the treatment and this was comparable to Experiment 1.

The lag in recovery may also be a consequence of 1,8-cineole biological activity. Speculation on a possible mode of action suggests either a direct effect of 1,8-cineole on gastric tissue, a systemic effect resulting from elevated blood levels of 1,8-cineole or its metabolite or an adverse effect on intestinal microorganisms. We have accumulated other evidence throughout this thesis to support the hypothesis that 1,8-cineole affects gastrointestinal function. Firstly, we observed in this experiment that the sudden introduction of 1,8-cineole had a dramatic affect on faecal output. Some possums ceased to defecate for a number of days when a diet of 4 % 1,8-cineole was introduced, plus, faecal weights were significantly reduced when defecation did occur. The number of possums defecating and the weight excreted increased once the 1,8-cineole was removed from the diet (Figure 10.9-C.).

Secondly, preliminary studies to obtain pharmacokinetic data on 1,8-cineole metabolism in possums were only moderately successful due to a double-peak phenomenom occuring in the blood profile of 1,8-cineole (reported in Appendix 1 - section A1.3.2). It is proposed that a negative feedback loop results from elevated blood concentrations of 1,8-cineole (or possibly its metabolites). Therefore, when 1,8-cineole blood concentrations reach a critical level, gastric function is affected, probably interupting gastric mobility. Consequently, no further absorption of 1,8-cineole occurs until gastric function resumes.

If this speculation is correct, reduced recovery of ingested 1,8-cineole during the early days of the treatment may be due to inhibited absorption of the daily intake of 1,8-cineole. A quantity of ingested food would remain in the gastrointestinal tract until metabolism reduced blood levels sufficiently to allow resumption of gastric function. Induction of metabolism would increase the clearance of 1,8-cineole, reducing the effect on gastric function, thereby increasing the amount of 1,8-cineole absorbed and excreted each day.

Introducing a diet containing 4 % 1,8-cineole reduced urine output. There was a consistent reduction in urine volume for all possums at the commencement of 1,8-cineole treatment. This was probably a direct result of reduced food intake. Urine pH measurements also decreased dramatically from 8.5 to 6.5. This was a direct result of 1,8-cineole metabolite excretion and reflects the acidity of the metabolites. pH measurements were consistent with those measured in Experiment 1 at 4 % 1,8-cineole (days 16 and 17).

10.4. Experiment 3 - Inhibition of 1,8-cineole ingestion

The aim of Experiment 3 was to attempt to effect a reduction in maximum 1,8-cineole intakes in possums and thereby alter the metabolite excretion pattern by pretreatment with probenecid, a glucuronyl transferase inhibitor (Abernathy *et al.* 1985) and inhibitor of renal tubular secretion of organic acids (Rammelkamp and Bradley 1943). Probenecid was incorporated into the diet of possum two days before 1,8-cineole was introduced and it was continued throughout the 1,8-cineole

treatment period. The 1,8-cineole treatment regime was the same as in Experiment 2.

Probenecid was an appropriate inhibitor of 1,8-cineole metabolism and excretion since glucuronidation was found to be important in the elimination of 1,8-cineole. Furthermore, the majority of 1,8-cineole metabolites would be expected to form organic anions at physiological pHs, therefore their renal secretion could also potentially be inhibited by probenecid. Probenecid has a large safe therapeutic concentration range in humans and was therefore considered to be a relatively safe agent to use in possums.

10.4.1. Methods

As there were no reports on the safety of administration of probenecid in possums in the literature, a preliminary trial of two possums for 4 days was undertaken to ensure tolerance and acceptability of the combined treatment. The remaining four possums were only treated after it was established that there were no evident adverse effects.

Possums were placed into metabolism cages on day 0 and fed the basal diet as in Experiments 1 and 2. Probenecid (*p*-[dipropylsulfamoyl] benzoic acid, purchased from Sigma Chemical Co, St Louis, Montana, USA) is a dry white powder, almost insoluble in water. It was incorporated into the diet by sprinkling onto a small portion and thoroughly mixing this through the remainder of the diet to produce a final concentration of 0.1 % (wet weight). This concentration ensured a daily intake ranging between 40 and 100 mg/kg (dependent on food intake) which was comparable to the dose used in humans studies (Abernathy *et al.* 1985). The probenecid diet was commenced on day 1 and continued for the remainder of the experiment. 1,8-Cineole (4 % wet weight) was introduced on Day 3 and was continued for 4 days.

Observational data was collected and urine analyses were performed in the same manner as described in Chapter 9 and used for Experiments 1 and 2.

10.4.2. Results

10.4.2.1. Dietary intake

Observational results for intake of food (and therefore probenecid and 1,8-cineole) are reported in Figure 10.17. Food and 1,8-cineole intakes were significantly reduced during the treatment period. Furthermore, food and 1,8-cineole intakes were significantly lower (72 and 68 % on days 3 and 6, respectively) in Experiment 3 compared to Experiment 2 (Anova (two factor with replication) $df = 1$, $F = 17.85$, $P < 0.001$; Tables 10.6 and 10.10).

Urine output was variable and there was no significant variation in mean urine output throughout Experiment 3 (Figure 10.18-A). Urine pH is reported in Figure 10.18-B and, as was observed in the previous two experiments, became more acidic during the 1,8-cineole treatment. pH measurements were higher than those measured in Experiment 2 (Anova (two factor with replication) $df = 1$, $F = 5.43$, $P = 0.02$).

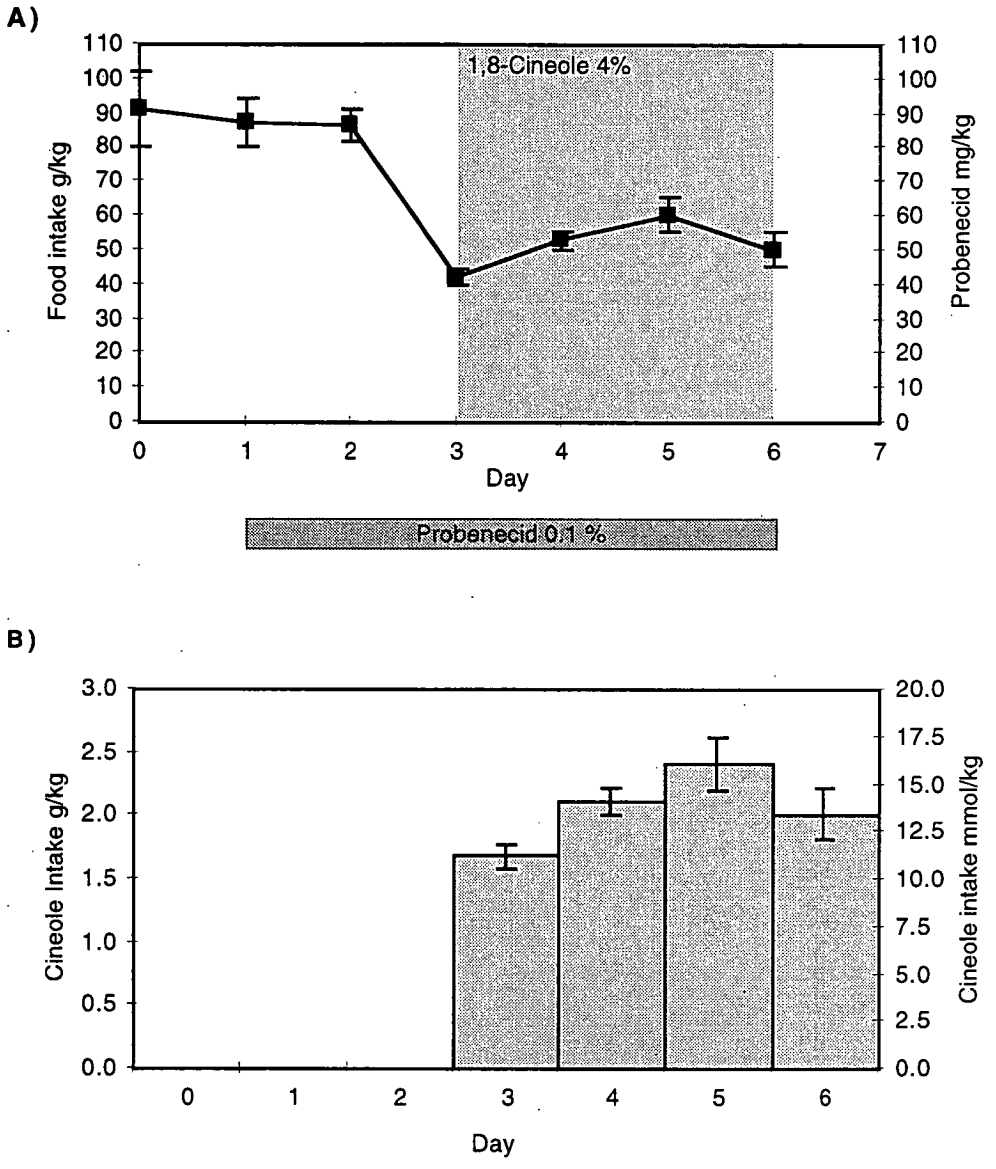


Figure 10.17. A) Food and probenecid intake (g/kg; mean ± se, n = 6) decreased with the introduction of the 1,8-cineole treatment (Anova (single factor) *df* = 6, F = 11.74, *P* < 0.001). The 1,8-cineole treatment period is indicated by shading. B) 1,8-Cineole intake (g/kg and mmol/kg).

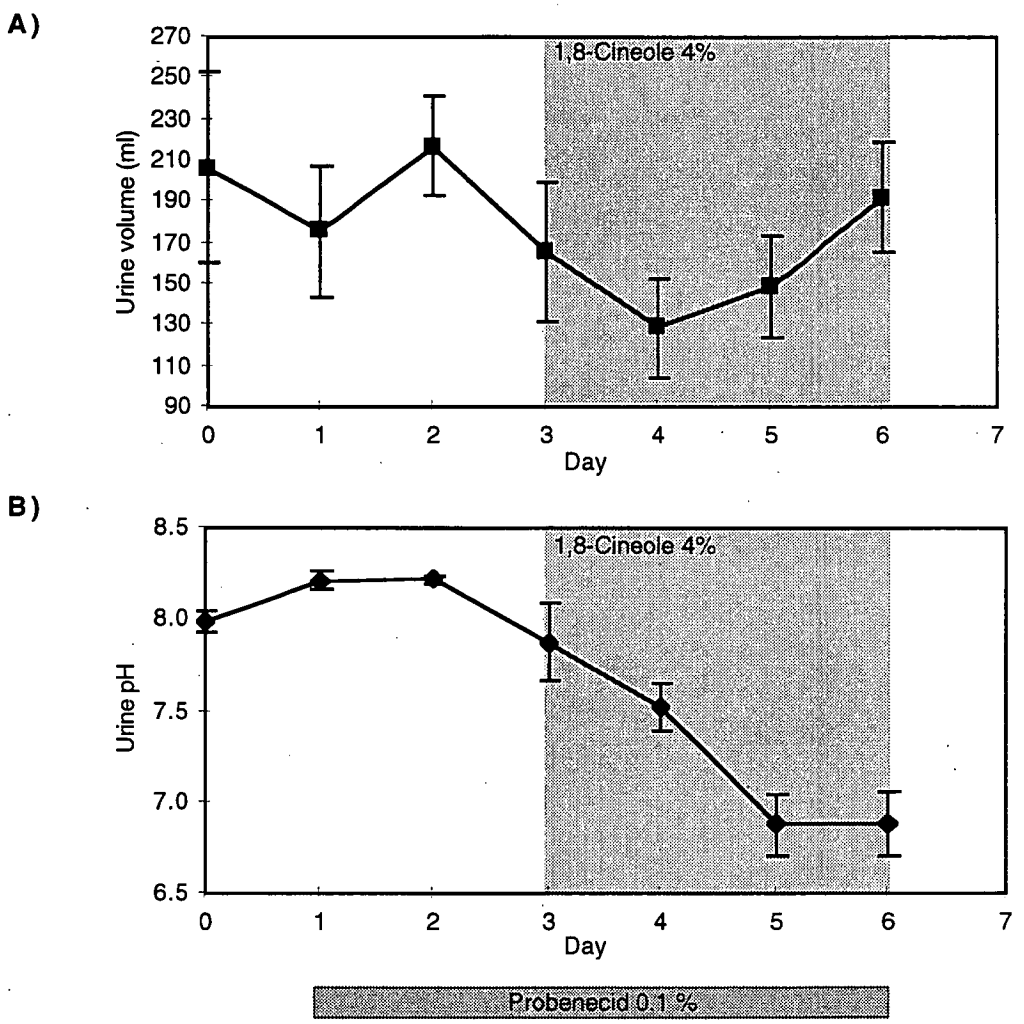


Figure 10.18. Urine data for each day of Experiment 3. A) Urinary output (ml) was variable with no significant difference in outputs throughout the treatment (*df* = 6, F = 1.02, *P* = 0.43) and B) urine pH decreased significantly during the treatment (*df* = 1, F = 5.43, *P* = 0.02). Values are reported as mean ± se (n = 6) and Anova (single factor) was used for statistical comparisons. The 1,8-cineole treatment period is indicated by shading.

10.4.2.2. Metabolite analyses

Urine samples from days 2, 3, 4 and 6 were analysed for urinary metabolites and glucuronic acid. The 1,8-cineole intakes for these days are summarised in Table 10.10 as well as the treatment regime for probenecid and 1,8-cineole. Data for individual possums are reported in Appendix 8 - Table A8.5.

Table 10.10. Dietary concentrations and intakes for 1,8-cineole and probenecid for urine sample days in Experiment 3 (n = 6).

	Day 2		Day 3		Day 4		Day 6	
	Mean	sd	Mean	sd	Mean	sd	Mean	sd
Additions to diet (% wet weight)								
probenecid	0.1		0.1		0.1		0.1	
1,8-cineole	0.0		4.0		4.0		4.0	
Probenecid intake								
mg/kg	87	5	42	2	53	3	50	5
1,8-Cineole intake								
g	0.0	0.0	6.3	1.5	7.9	1.9	7.4	1.6
mmol	0.0	0.0	40.9	9.9	51.6	12.2	47.8	10.7
mmol/kg	0.0	0.0	11.5	1.9	14.5	2.2	13.9	4.5

As in the previous experiments, eighteen metabolites were identified and quantified. Molar recoveries for individual metabolites are shown in Figure 10.19. Although the amounts excreted change for each day, the relative abundances of each metabolite can be easily visualised.

Comparing the pattern of metabolite excretion between Experiment 2 and 3 revealed that some of the more highly oxidised metabolites were more abundant in Experiment 3 in the early days, particularly the dihydroxycineole metabolite Ci 11 and the hydroxy cineolic acids Ci 8, 9 and 15. On subsequent days, the metabolite pattern changed and was finally very similar to Experiment 2, with only minor variations in relative abundances.

The fractional recovery of 1,8-cineole was initially low but increased throughout the 1,8-cineole treatment, following a similar pattern to that seen in Experiment 2 (Table 10.11). The fractional recovery of total metabolite was significantly greater than the recovery of free metabolites for each day, which is also in agreement with the previous experiments.

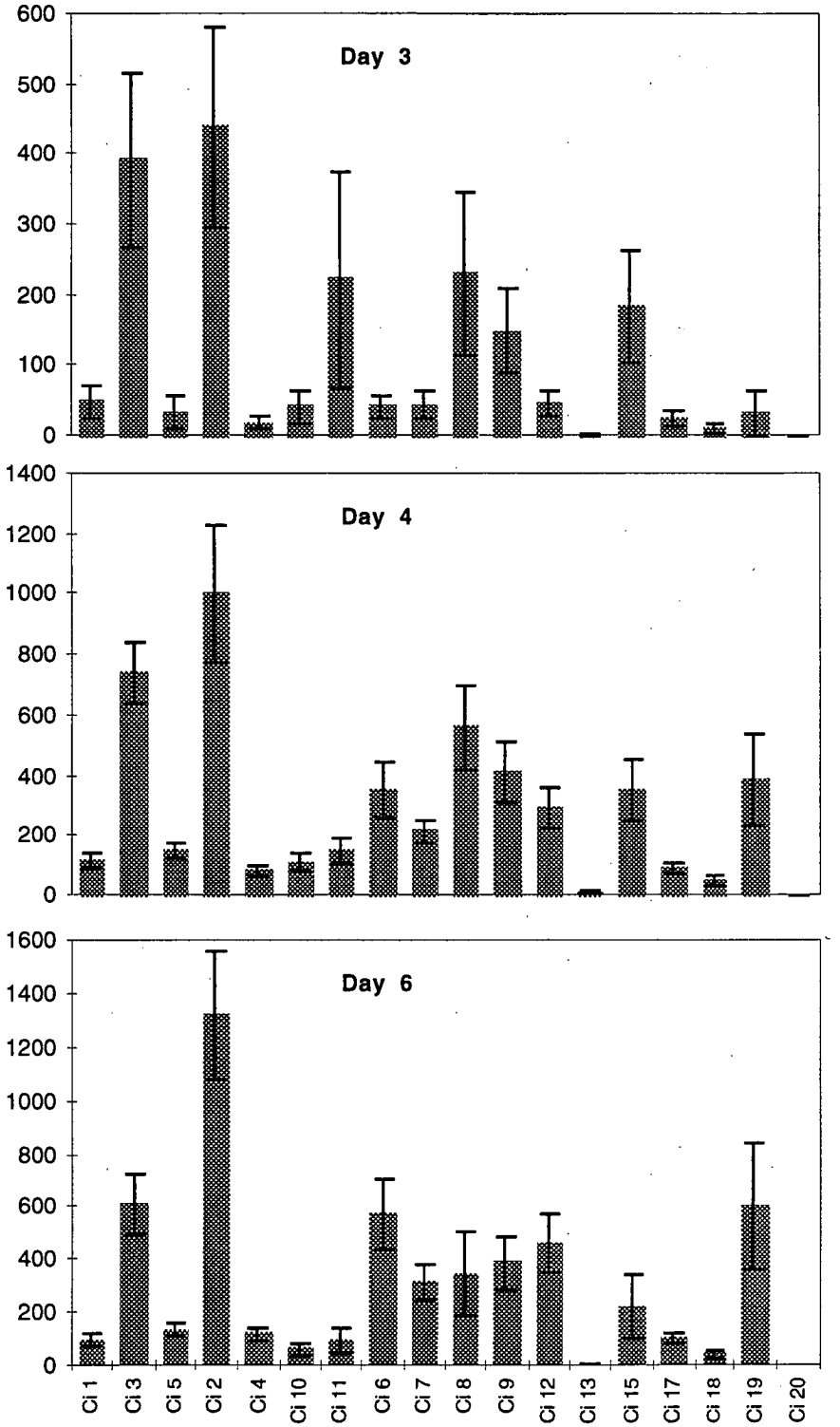


Figure 10.19. Individual 1,8-cineole metabolite recoveries (µmol/kg), after hydrolysis, for each metabolite analysis day. Metabolites are identified in Chapter 9 - Figure 9.6 and Table 9.3 and are reported as mean ± se (n = 6).

Figure 10.11. Fractional recovery of 1,8-cineole intakes as urinary metabolites (n = 6) in Experiment 3.

	Fraction of administered dose					
	Day 3		Day 4		Day 6	
Possum	Free	Total	Free	Total	Free	Total
Mean ± sd	0.12 ± 0.10	0.17 ± 0.13	0.21 ± 0.05	0.37 ± 0.10	0.31 ± 0.16	0.46 ± 0.28

Hydrolysis resulted in an increase in metabolite recovery each day (Anova (two factor with replication) $df = 1, F = 5.19$ $P = 0.03$).

Total recovery of metabolites increased significantly throughout the treatment period (Anova (single factor) $df = 2, F = 3.57$, $P = 0.05$).

Figure 10.20 shows the pattern of excretion of metabolite groups as their percentage of recovered 1,8-cineole. The broad pattern of metabolite excretion is again similar to that observed in Experiment 2, with the hydroxycineoles being the major group of metabolites on the first day of the treatment. However, hydroxy cineolic acids dominated the metabolite profile after two days, indicating induction of enzymes responsible for more extensive oxidation.

The molar recoveries of both free and total metabolites in each group are shown in Figure 10.21. The difference between total and free represents hydrolysable conjugation. Therefore hydrolysis resulted in significant increases in the overall recovery of metabolites, hydroxycineoles and cineolic acids. Recovery of dihydroxycineoles and hydroxy cineolic acids did not increase.

Free and total recoveries for individual metabolites are shown in Figure 10.22 and and the associated statistical comparison between the two measurements are reported in Table 10.12. Individual metabolites generally reflected the pattern of conjugation of their respective groups, except for the hydroxy cineolic acid, Ci 18, which was extensively conjugated. Again the hydroxycineoles underwent the greatest degree of oxidation.

The fractions of individual hydroxycineoles, cineolic acids conjugated, plus the total of all metabolites, are presented in Table 10.13. For Ci 2, 3 and 5 the fraction excreted in the conjugated form increased throughout the treatment but did not affect the fraction of total metabolites conjugated. The fraction of Ci 4 also increased throughout but this was not significant.

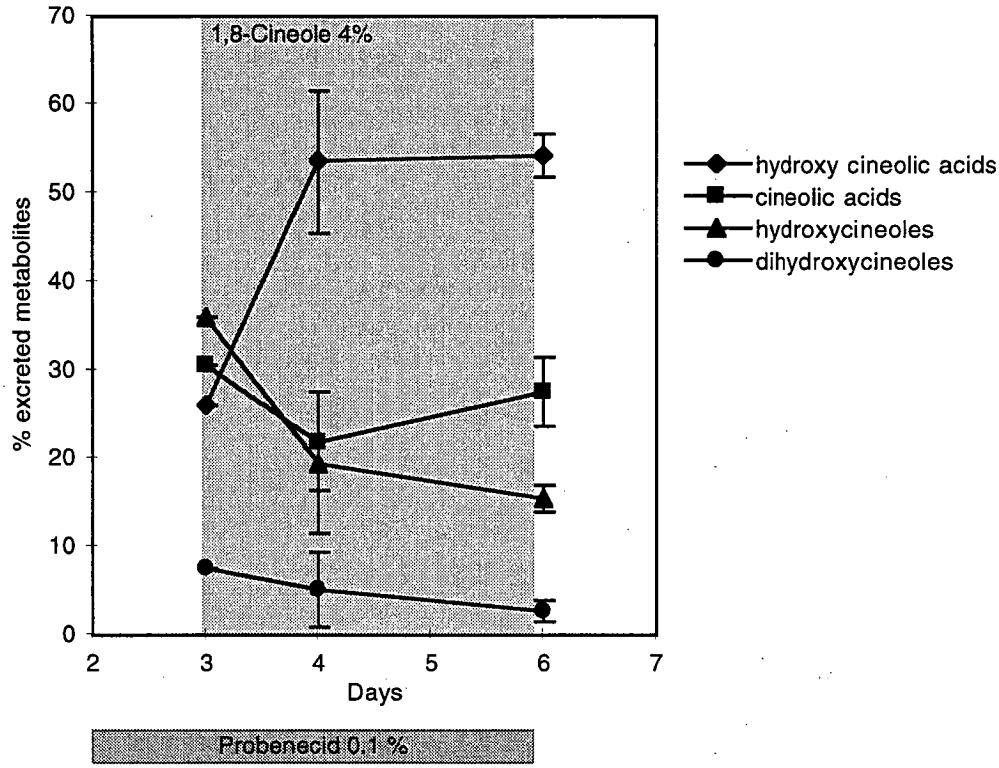


Figure 10.20. Recovery of 1,8-cineole metabolite groups as percentage of total metabolite recovery for brushtail possums in Experiment 3 (mean ± se, n = 6). The 1,8-cineole treatment is indicated by shading and the probenecid treatment is indicated by the bar at the base of the graph.

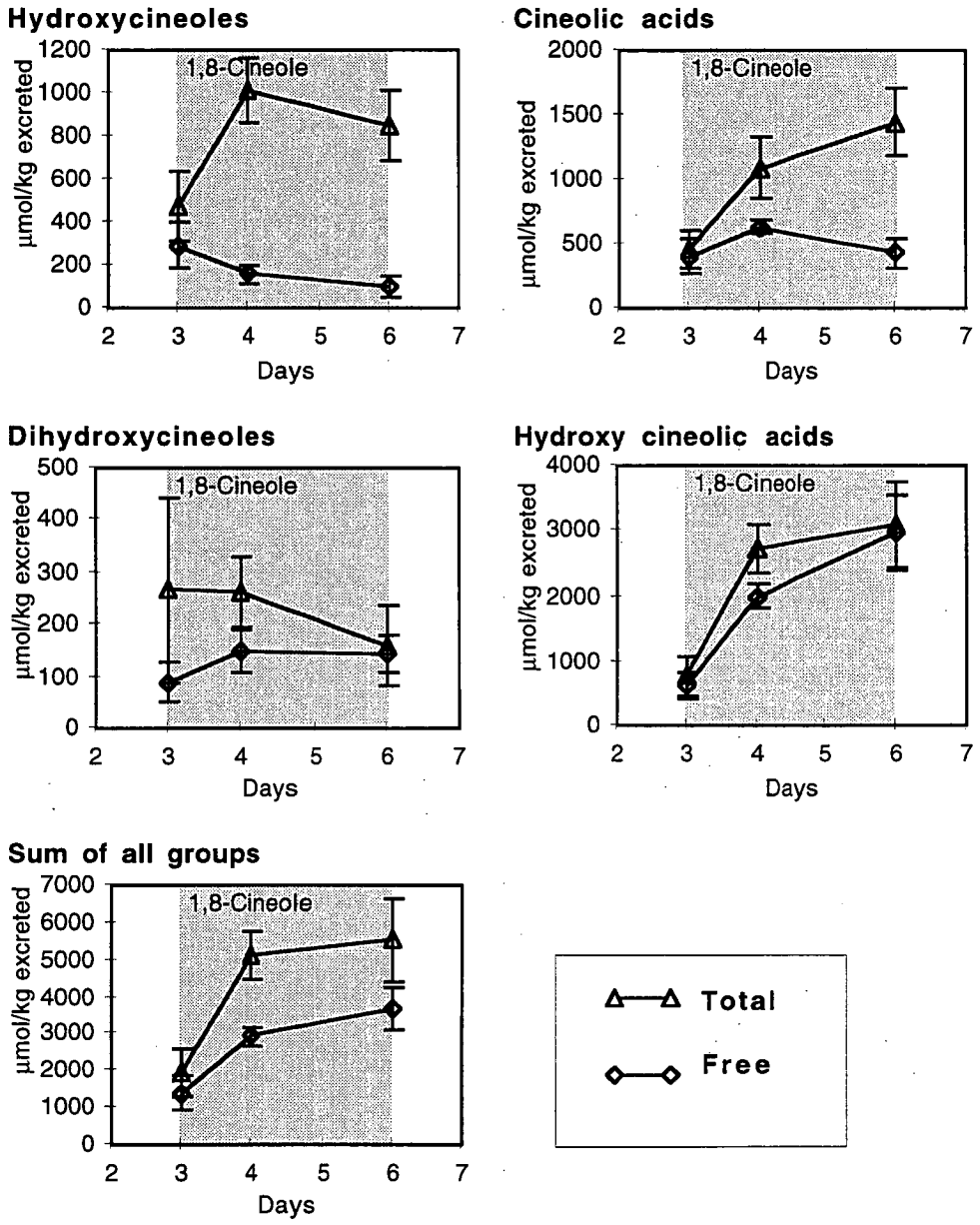
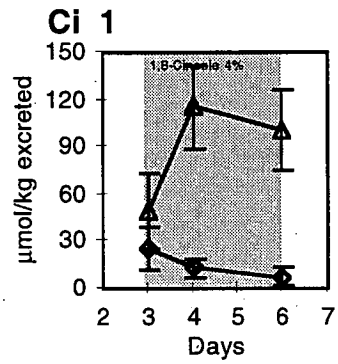
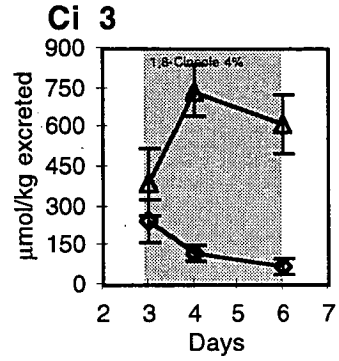
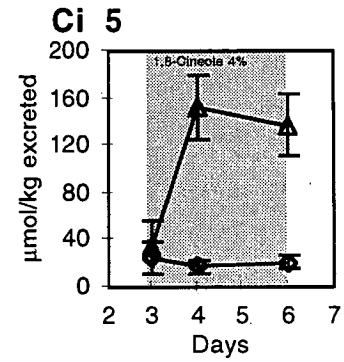


Figure 10.21. Comparison of free and total metabolites for each metabolite group in Experiment 3. Values are µmol/kg (mean ± se, n = 6). The 1,8-cineole treatment period is indicated by shading and probenecid (0.1 %) was also included in each day's diet. Hydrolysis increased the recovery of hydroxycineoles, cineolic acids and total metabolite recovery (Anova (two factor with replication) df = 1, F = 35.3, P < 0.001; df = 1, F = 12.75, P = 0.001 and df = 1, F = 7.89, P = 0.01, respectively). Recovery of dihydroxycineoles and hydroxy cineolic acids did not increase with hydrolysis (Anova (two factor with replication) df = 1, F = 2.06 P = 0.16 and df = 1, F = 1.26, P = 0.27, respectively).

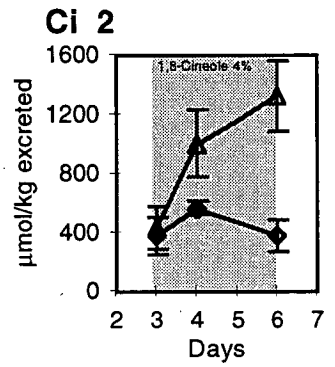
1) Hydroxycineoles

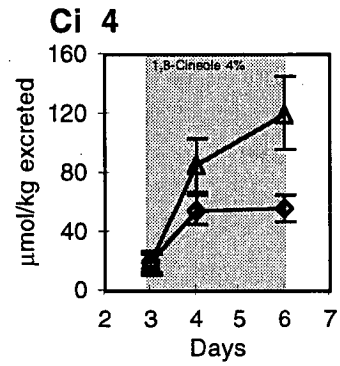




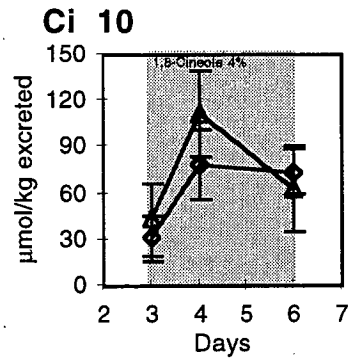


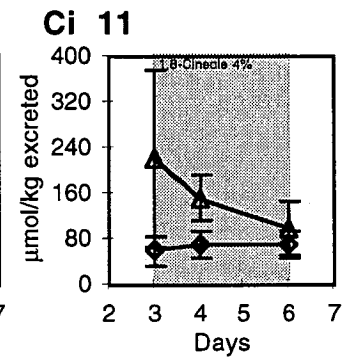
2) Cineolic acids



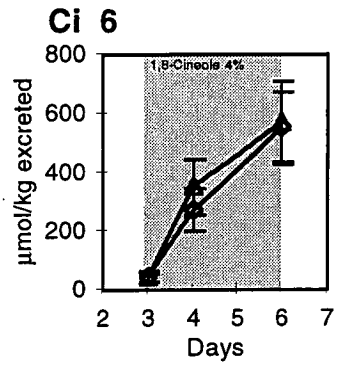


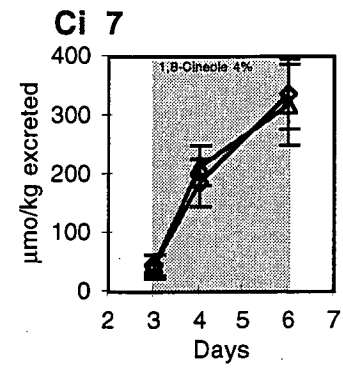
3) Dihydroxycineoles

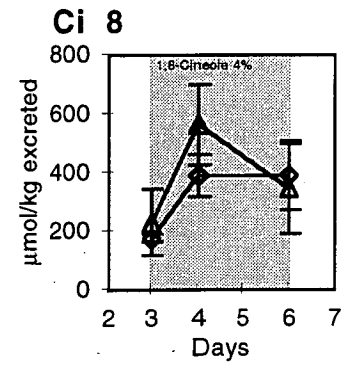




4) Hydroxy cineolic acids







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4) continued.

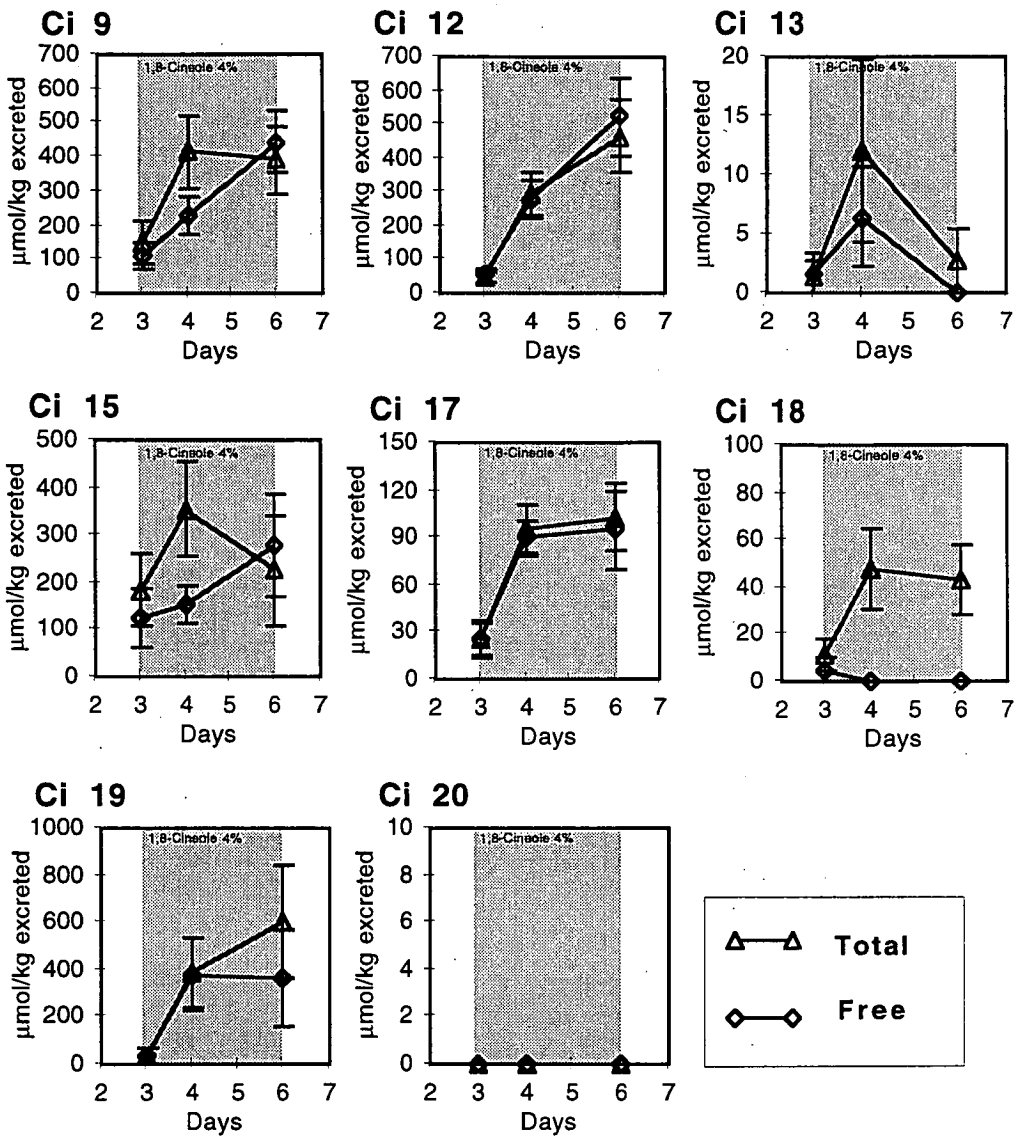

Figure 10.22. Comparison of free and total metabolite recoveries for individual metabolites in Experiment 3. Values are µmol/kg (mean ± se, n = 6). Anova comparisons of free and total recoveries for each metabolite are reported in Table 10.12. No Ci 20 was detected in any samples.

Table 10.12. Statistical comparison of total and free metabolites for individual metabolites shown in Figure 10.22.

Metabolite	Anova two factor with replication (n = 6)		
	df	F value	*P* value
Hydroxy cineoles			
Ci 1	1	21.91	<0.001
Ci 3	1	35.58	<0.001
Ci 5	1	28.5	<0.001
Cineolic acids			
Ci 2	1	12.8	0.001
Ci 4	1	7.02	0.01
Dihydroxycineoles			
Ci 10	1	0.37	0.55
Ci 11	1	2.51	0.12
Hydroxy cineolic acids			
Ci 6	1	0.22	0.64
Ci 7	1	0.01	0.92
Ci 8	1	0.4	0.53
Ci 9	1	0.79	0.38
Ci 12	1	0.08	0.49
Ci 13	1	0.73	0.4
Ci 15	1	0.93	0.34
Ci 17	1	0.11	0.74
Ci 18	1	15.89	<0.001
Ci 19	1	0.41	0.53
Ci 20[1]	1	0	1

[1]Ci 20 was not detected in this experiment. Its absence is probably due to the complete deterioration of its chromatography.

Table 10.13. Fraction of metabolites excreted as hydrolysable conjugates for the two metabolite groups that underwent the majority of conjugation plus all metabolites grouped together (mean ± sd, n = 6).

	Day 3	Day 4	Day 6	Statistical comparison[1]	
	Fraction of metabolites conjugated (mean ± sd)			F	*P* -value
Hydroxy cineoles					
Ci 1	0.49 ± 0.18	0.89 ± 0.04	0.79 ± 0.16	2.15	0.15
Ci 3	0.47 ± 0.09	0.83 ± 0.03	0.90 ± 0.05	9.14	0.004
Ci 5	0.41 ± 0.20	0.90 ± 0.09	0.85 ± 0.07	4.3	0.03
Cineolic acids					
Ci 2	0.21 ± 0.21	0.40 ± 0.25	0.68 ± 0.25	6.13	0.01
Ci 4	0.16 ± 0.16	0.28 ± 0.19	0.47 ± 0.26	3.36	0.06
Sum all	0.30 ± 0.28	0.41 ± 0.08	0.34 ± 0.18	0.54	0.59

[1]Anova (single factor) comparing the fraction of conjugated metabolites between the three sample days for the most conjugated metabolites (*df* = 2).

Glucuronic acid increased throughout the treatment (Figure 10.23) and was comparable to that measured in Experiment 2 (Anova two factor with replication $df = 2$, $F = 2.61$, $P = 0.12$). Once again there was a good correlation between 1,8-cineole intake and urinary glucuronic acid excretion ($df = 17$, $R^2 = 0.73$, $P < 0.001$). Values for glucuronic acid and conjugated metabolites are recorded for individual possums in Appendix 8 - Table A8.6.

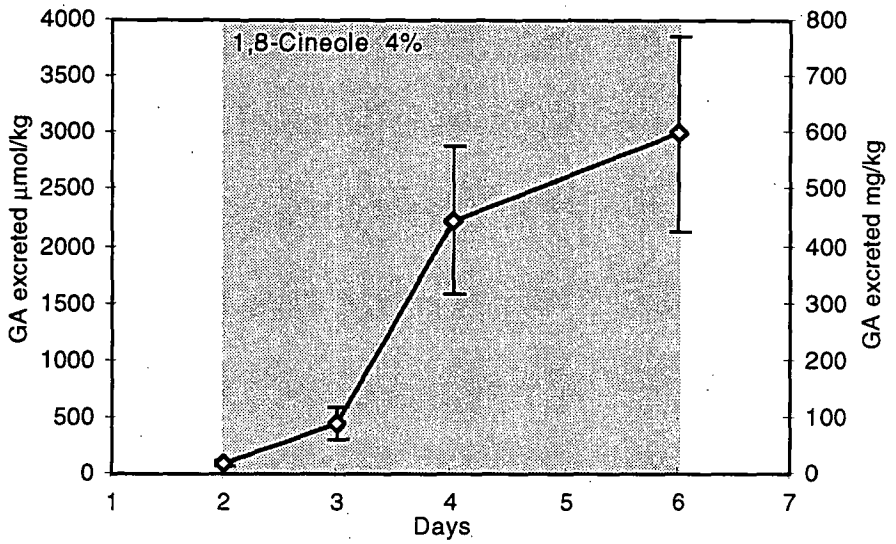

Figure 10.23. Urinary glucuronic acid (GA) excretion (µmol/kg and mg/kg; mean ± se, n = 6) by possums in Experiment 3 increased throughout the 1,8-cineole treatment period (Anova (single factor) $df = 3$, $F = 6.45$, $P = 0.003$).

10.4.3. Discussion

Similar trends were found in feeding data and metabolite analyses to Experiment 2 and will not be repeated in this discussion. Instead, data from Experiment 3 will be compared with Experiment 2 as control data. The same possums were used in both experiments and although two months separated Experiment 2 and 3, each experiment was conducted during the winter months (June and August 1997), and possum weights, diet and condition were comparable at the commencement of each experiment.

A significant reduction in 1,8-cineole ingestion in possums pre-treated with the uricosuric agent, probenecid, has been demonstrated. Most observational parameters that were at variance with Experiment 2 can generally be explained in relation to the reduced 1,8-cineole intake. Food intake was directly proportional to 1,8-cineole ingestion and was also significantly reduced. Urinary pH was also higher in Experiment 3 as a result of the lower acid metabolite load requiring excretion.

Comparison of urinary metabolite analyses also revealed some variations in the pattern of metabolism between the two experiments.

Fractional recoveries of ingested 1,8-cineole in each experiments are summarised in Figure 10.24. Overall recovery was greater in Experiment 3, yet the slopes of the regression equations are almost equal. This indicates that the rate of recovery increase was the same for both experiments. The higher fractional recovery in Experiment 3 may result from the variations in either of the variable parameters of the following equation:

$$\text{Fraction recovered} = \sum\text{metabolites recovered (}\mu\text{mol/kg)/1,8-cineole intake (}\mu\text{mol/kg)}$$

1,8-Cineole intake was shown to be less than that in Experiment 2. Therefore the fraction of 1,8-cineole recovered increased as a consequence of there being no, or a proportionally lower, increase in the molar excretion of metabolites, despite the lower intake of 1,8-cineole.

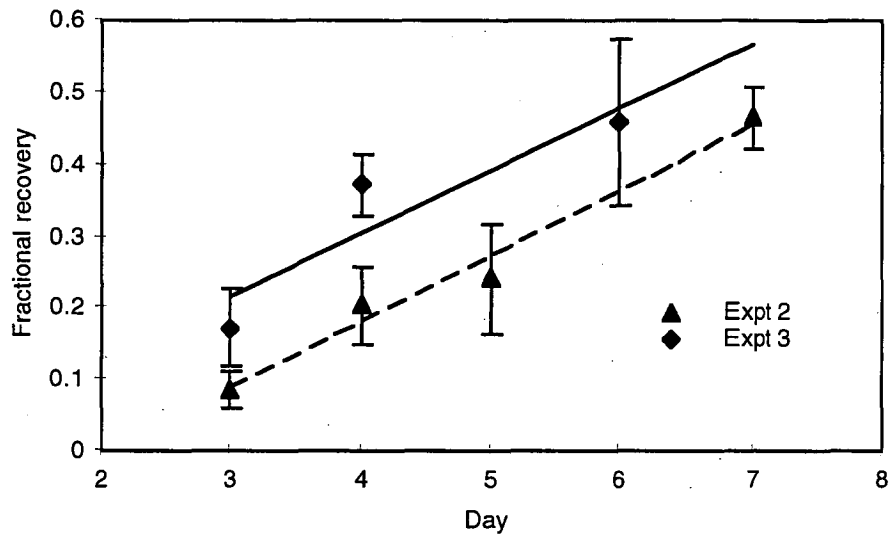

Figure 10.24. Fractional recovery of 1,8-cineole as total metabolites for Experiments 2 and 3. Regression equations are $y = 0.092x - 0.185$, $R^2 = 0.98$ and $y = 0.088x - 0.047$ $R^2 = 0.84$ for Experiment 2 (hashed line) and 3 (solid line), respectively.

The mechanism by which probenecid could affect 1,8-cineole ingestion is unclear due to its multiple modes of action. Probenecid was chosen as a potential inhibitor because it has been reported to inhibit the formation of ether glucuronides, a significant route of elimination of hydroxycineoles and cineolic acids (Abernathy *et al.* 1985), and competitively inhibit renal tubular secretion of acid or glucuronide metabolites (Rammelkamp and Bradley 1943). Competitive inhibition of renal secretion is unlikely since the molar concentration of acid metabolites was very much greater than the the molar amount of probenecid ingested. Furthermore, the total molar amounts of metabolites excreted between the two experiments were comparable which also suggests that there was no inhibitory effect on renal excretion

of metabolites (Figure 10.12. and 10.21).

There was also little evidence of inhibition of glucuronide formation. The fraction of metabolites excreted as glucuronides was comparable between both Experiment 2 and 3 (Table 10.9 and 10.13). However, fewer of the hydroxy cineolic acids showed conjugation in Experiment 3 (Table 10.12). As these were the most polar metabolites they would still be readily excreted without glucuronidation and therefore be the least affected by reduced glucuronidation.

The pattern of excretion on day 3 of Experiment 3 suggested that metabolites had undergone significantly more oxidation than those excreted on day 3 of Experiment 2 (Figure 10.10 and 10.19). Residual enzyme induction from Experiment 2 could not be responsible for the variation as the possums had been fed a fruit and vegetable diet for 2 months between experiments. This variation is possibly associated to the probenecid treatment.

10.5. Conclusions

1,8-Cineole metabolism was extremely complex in brushtail possums. A large array of eighteen metabolites were found in this species. The complexity of the metabolite pattern apparently arises out of the possums' ability to oxidise most of the carbons on the 1,8-cineole molecule, including the ring carbons (Carman and Klika 1992; Carman *et al.* 1994; Carman and Rayner 1994; Carman and Garner 1996).

Although 1,8-cineole results in a greater number of metabolites, the detoxification strategy is similar to that employed for *p*-cymene (Chapter 4). In both cases, many metabolites were excreted, encompassing all degrees of oxidation. Glucuronidation was also very important in the elimination of the less oxidised metabolites, although in both cases there appears to be some conjugation of more polar metabolites.

It has been demonstrated that brushtail possums are able to maintain their body weight while consuming a diet containing high concentrations of a single PSM. Although at high dietary concentrations a threshold in 1,8-cineole intake was apparent in some possums, there was no evidence from the pattern of metabolites excreted that saturation of metabolism occurred. Therefore it seems that brushtail possums can readily ingest and detoxify challenging levels of 1,8-cineole.

1,8-Cineole was selected as a common representative eucalypt terpene for the study of chronic detoxification. Since the metabolic strategy employed to detoxify another terpene, *p*-cymene, was similar, it is therefore probable that brushtail possums employ a similar strategy in the detoxification of most terpenes in their diet. In summary, multiple oxidation pathways are employed, producing a large number of metabolites. The majority of the more extensively oxidised metabolites are excreted directly via the kidneys while the less oxidised metabolites are extensively conjugated with glucuronic acid before renal excretion. This strategy results in an ability to ingest, detoxify and rapidly excrete large quantities of what has been considered a toxic compound.

These findings do not directly support the widely-regarded hypothesis that

generalist herbivores select their leaf diet to ensure small quantities of a wide range of PSMs are ingested to avoid saturation of enzymatic detoxification pathways (Freeland and Janzen 1974). This hypothesis was partly founded on the observation that possums do not maintain their body weight when fed a diet consisting of only one *Eucalyptus* species (Freeland and Winter 1975). This was attributed to the concentration of relatively few PSMs in a single species and assumed that detoxification pathways would be saturated.

The inability of Freeland and Winter's (1975) possums to thrive can probably be reinterpreted as a result of a complex interrelationship between a nutritionally poor diet plus a detoxification challenge from a mixture of PSM. It must be remembered that the basal diet fed to our possums was nutritionally balanced and of high quality compared to leaf. Furthermore, possums were challenged with only a single compound. Therefore detoxification conditions were optimal, and competition for enzymes or metabolic substrates such as glucuronic acid was minimal.

CHAPTER 11

CHRONIC INGESTION OF 1,8-CINEOLE IN KOALAS

11.1. Introduction

Urine from the *p*-cymene dosing experiments in koalas was re-analysed for 1,8-cineole metabolites. Since koalas were feeding on *E. cephalocarpa*, the terpene profile of this species was analysed and found to be > 90 % 1,8-cineole, analysis of the urine provided a snapshot of the metabolic fate of 1,8-cineole during chronic dosing. The amount of 1,8-cineole ingested was determined from the amount of leaf consumed and the measured concentration of 1,8-cineole in leaf samples.

Understanding the metabolic fate of 1,8-cineole in koalas allows us to make a general comparison with its metabolism in brushtail possums and to consider the findings, along with those for *p*-cymene metabolism, from an ecological perspective.

Consideration was also given to other leaf derived metabolites occurring in koala urine. Of particular interest were glucuronide conjugated compounds and koala urine was analysed directly to investigate the source of these.

11.2. Methods

11.2.1. Dosing and urine collection

The experimental procedure for *p*-cymene dosing of six male koalas was detailed in Chapter 7. Urine samples collected from 24 - 48 h after the 1.49 mmol/kg *p*-cymene dose were used for 1,8-cineole metabolite analyses. Koalas had been feeding on *E. cephalocarpa* for at least three days. There was minimal interference from *p*-cymene metabolites in these samples as the majority of the *p*-cymene dose had been excreted in the previous 24 h.

Koalas were offered daily, fresh leaf from one of twelve trees. Leaf was picked on a weekly basis from purpose grown plantations on Philip Island. The amount of 1,8-cineole ingested was proportional to the amount of leaf consumed. Daily leaf intakes were measured and the amount of 1,8-cineole ingested calculated from the measured leaf concentration.

Urine volume and pH measurements were reported in Chapter 7.

11.2.2. Leaf analysis

Leaf samples from each of the twelve trees were collected and frozen at -18°C until analysed. Dry mass (DM) was determined on samples of fresh leaf by drying at 70°C to a constant weight.

The ethanol extractable component of leaves was analysed to determine the terpene profile and 1,8-cineole content of *E. cephalocarpa* (Chapter 7 - section 7.2.2). Verification of terpene content by steam distillation of the volatile component of leaf samples from two trees confirmed effectiveness of the ethanol extraction method.

For steam distillation, an accurately weighed sample of frozen leaf (approximately 30 g) was roughly chopped into smaller fragments and placed into a 1 l flask with 500 ml of distilled water. The flask was fitted to a volatile oil steam distillation apparatus (configured for oil lighter than water) and a secondary condenser added. The mixture was allowed to reflux for two and a half hours, at which time no further oil was being accumulated in the condenser. The apparatus was allowed to cool and the oil decanted. The condenser apparatus was rinsed with ether (10 ml) which was combined with the oil fraction. The leaf mixture was reheated and allowed to reflux for a further two hours and any trace of oil fraction removed as before. Magnesium sulphate (1 g) was added to dry the ether mixture before filtering through Whatman 2 filter paper. Ether was removed on a rotary evaporator and the final oil yield weighed. 1,8-Cineole concentration for frozen wet and dry mass were calculated.

11.2.3. Urine metabolite analysis

Urine and faecal samples were analysed for both free and total 1,8-cineole metabolite levels and glucuronic acid as described in Chapter 9.

Preliminary investigations into other leaf derived glucuronide metabolites by LC/MS was also detailed in Chapter 9.

11.3. Results

11.3.1. Leaf analyses

Terpene profile. The GC/MS of a sample ethanol extract of *E. cephalocarpa* is shown in Figure 11.1. Clearly, the major component was 1,8-cineole. Detectable amounts of limonene, *p*-cymene (estimated amounts reported in Chapter 7 - section 7.3.4) and α- and β-pinene were also present. There were very few peaks occurring where sesquiterpenes and larger molecules would be expected to elute (Rt = 8 - 20 min).

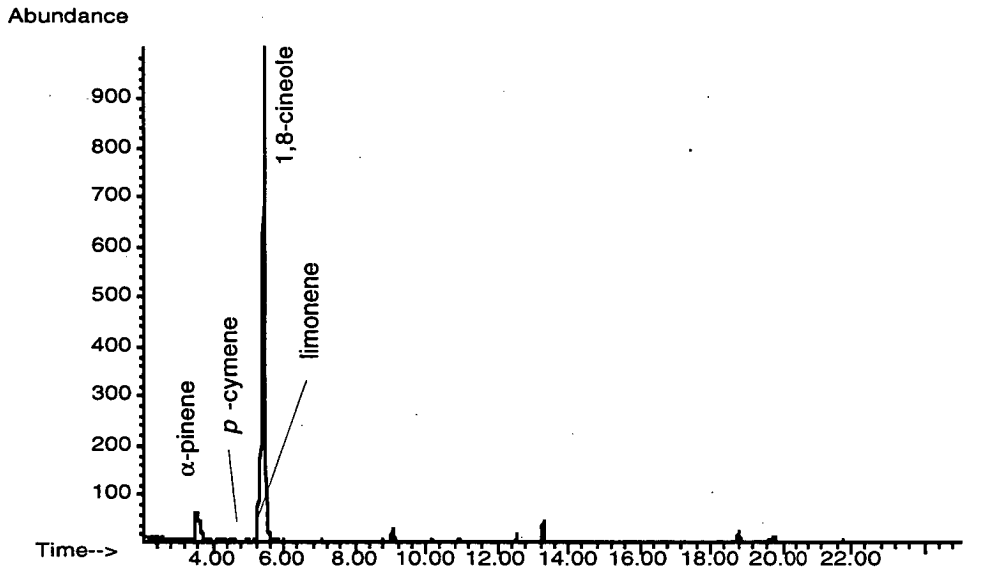

Figure 11.1. GC/MS of ethanolic extract of *E. cephalocarpa* leaf. This was the chosen diet for koalas during *p* -cymene dosing experiments. 1,8-Cineole was the predominant terpene with detectable amounts of limonene, α-pinene and *p* -cymene. There were no sesquiterpenes detected.

1,8-Cineole leaf concentrations. Table 11.1 reports calculated 1,8-cineole concentrations for both frozen wet and dry mass (DM) of leaf samples from each tree. Steam distillation recoveries from tree # 1 and 5 are also recorded. The dry mass (DM) for leaf samples from each tree are also reported.

Estimation of 1,8-cineole leaf concentrations is subject to unpredictable natural variation. Terpene profile and content can be variable at many scales, including leaves within the same tree. Therefore, extrapolating leaf concentrations from one leaf sample from a tree to all leaf collected from that tree will only provide an estimate. Therefore the amount of 1,8-cineole given in the following section is nominal.

1,8-Cineole ingestion. Daily consumption of leaf (fresh wet weight) and estimated 1,8-cineole intake for each day of the *p*-cymene dosing experiments were reported in Chapter 7 - Table 7.3. Estimated 1,8-cineole intakes durine the 24 h which urine was collected for 1,8-cineole metabolite analysis are summarised in Table 11.2.

Table 11.1. 1,8-Cineole concentration in frozen *E. cephalocarpa* l eaf from each feed tree plus steam distillation of two leaf samples.

E. ceph tree #	DM[1] % total	Concentration of 1,8-cineole (%)			
		Ethanol extract		Steam distillation	
		wet (frozen)	dry	wet	dry
1	42.4	1.15	2.71	1.15	2.71
2	46.6	1.58	3.39		
3	43.0	1.29	3.00		
4	41.3	1.34	3.23		
5	40.6	0.84	2.06	0.80	1.97
6	44.7	0.71	1.59		
7	39.8	0.60	1.51		
8	42.1	0.83	1.98		
9	38.8	0.92	2.36		
10	41.1	1.14	2.78		
11	41.6	0.90	2.15		
12	41.7	1.55	3.61		
Mean	42.0	1.07	2.53		
sd	2.1	0.32	0.70		

[1]DM = dry mass of leaves.

nb. Wet (frozen) weight may be less than wet (fresh) weight due to sublimation of water. Thus overestimation of 1,8-cineole intake may result.

Table 11.2. Summary of 1,8-cineole intakes for days urine samples were analysed for 1,8-cineole metabolites.

Koala	Koala weight	1,8-Cineole intake		
	kg	g	mmol	mmol/kg
1	9.89	3.05	19.78	2.00
2	8.34	1.65	10.73	1.29
3	9.74	1.69	10.94	1.12
4	8.05	5.05	32.77	4.07
5	9.30	4.05	26.28	2.83
6	7.40	3.45	22.38	3.02
Mean	8.79	3.15	20.48	2.39
sd	1.01	1.33	8.66	1.13

11.3.2. Urine analysis results

Urinary 1,8-cineole metabolites. Seven metabolites were identified and quantified. They were Ci 2 and 4 (9- and 7-cineolic acid), Ci 3 and 5 (9- and 7-hydroxycineole), Ci 13 and 17 (7-hydroxy-9-cineolic acid and 9-hydroxy-7-cineolic acid) and Ci 22 (7,9-dicineolic acid). The fraction of the estimated dose recovered was 1.3 ± 0.4 and 1.4 ± 0.4 (mean ± sd) for free and total measurements respectively, which although high was consistent between the two measurements.

Urine output was 101 ± 41 ml (mean ± sd) and pH was 5.4 ± 0.2 (mean ± sd) for the urine samples analysed (n = 6).

Molar recoveries for each metabolite are reported in Appendix 9 - Table A9.1 for individual koalas and the results reported in this chapter were derived from those values. The abundance of each metabolite is shown in Figure 11.2 for both free and total measurements. Overall, hydrolysis did not increase the recovery of total metabolites, but the recovery of the minor, less oxidised, metabolites Ci 2, 3, 4 and 5 increased significantly .

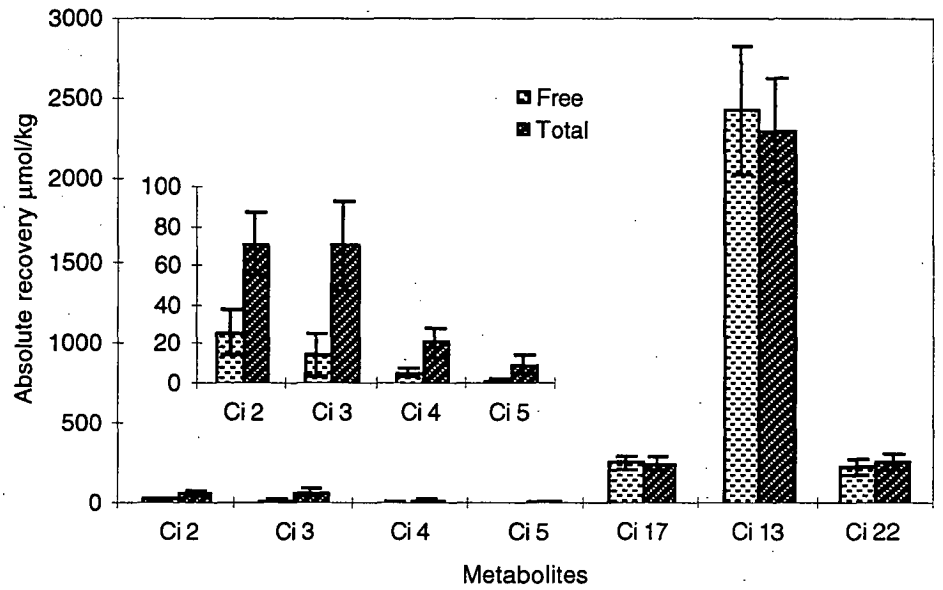

Figure 11.2. Molar recoveries (µmol/kg) of free and total levels of 1,8-cineole metabolites in koala urine (mean ± se, n = 6). Overall, hydrolysis did not increase the total recovery (Anova (two factor with replication) *df* = 1, F = 0.004, *P* = 0.95). Inset: minor metabolite recoveries are scaled up to show that hydrolysis did increase the recovery of Ci 2, 3, 4 and 5 (Anova (two factor with replication) *df* = 1, F = 9.01 *P* = 0.005).

Each metabolite group was reported as the percent of total recovered metabolites for both free and total measurements (Table 11.3). About 94 % of total recovered metabolites had acquired three or four oxygens during oxidation, resulting in highly polar metabolites. The hydroxy cineolic acids dominate the metabolite profile. The dicineolic acid metabolite is the most oxidised 1,8-cineole metabolite of all and is the second most significant metabolite accounting for almost 10 % of the recovered dose.

Table 11.3. Excretion of free, conjugated and total 1,8-cineole metabolites expressed as a percentage of total recovered metabolites in koalas eating *E. cephalocarpa* leaves.

Metabolite group	Percent (mean ± sd) of total urinary metabolites (n = 6)		
	Free	Conjugated	Total
Hydroxycineoles	0.4* ± 0.6	2.1 ± 1.3	2.5* ± 1.5
Cineolic acids	0.9* ± 0.7	2.3 ± 1.6	3.3* ± 1.5
Hydroxy cineolic acids	88.2 ± 14.1	3.3 ± 6.3	85.4 ± 2.1
Dicineolic acid	7.9 ± 2.6	0.9 ± 0.8	8.8 ± 2.6
Sum	97.4 ± 14.6	6.7 ± 10.2	100

*Comparison of free and total metabolite recovery, $P < 0.05$ (Student's paired t -test).
Each value is the mean ± sd of individual koalas. Metabolites were grouped according to oxidation and free and total metabolites were determined, respectively, before and after hydrolysis, and the difference was considered to be due to the conjugated metabolites. For some metabolites free levels were slightly greater than total, and the value of the difference was considered to be "0".
Fractional recovery of 1,8-cineole was 1.3 ± 0.4 (mean ± sd).

Glucuronic acid. Glucuronic acid measurements for all urine collections from *p*-cymene dosing experiments are reported in Chapter 7 - Table 7.4. The amount of glucuronic acid measured in urine samples analysed for 1,8-cineole metabolites was 1.15 ± 0.78 mmol/kg (223 ± 151 mg/kg). 1,8-Cineole conjugated metabolites accounted for only about 10 % of this value (sum of conjugated metabolites was 125 ± 91 µmol/kg).

Identification of glucuronides. MS and MS/MS data for non-1,8-cineole derived peaks in koala urine were reported in Chapter 9. The molecular weights of the major glucuronides (A - F), and their respective aglycones, were reported in Chapter 9 - Table 9.9. The molecular weights of the aglycones would suggest that they were small aromatic or phenolic molecules. Four structures are possible for the aglycone with molecular weight of 108 and they are *ortho-*, *meta-* and *para*-methyl phenol and benzyl alcohol. Nine isomers of hydroxy benzyl alcohol, catechol, resorcinol and hydroxy quinol are possible and correspond to the aglycone molecular weight of 124. The aglycone with molecular weight of 122 is likely to be benzoic acid. Those aglycones with larger molecular weights have a large number of possible structures, however, gallic acid, the building block of hydrolysable tannins, has a molecular weight of 170 and may account for glucuronide F (Chapter 9 - Figure 9.9 and Table 9.9). The abundances of these peaks suggest that they are the major glucuronide components of the urine. Further investigation into the source of glucuronides is required.

11.3.3. Faecal metabolites

No 1,8-cineole metabolites were detected in the faecal extracts analysed in Chapter 7. However a small amount of unchanged 1,8-cineole was detected.

11.4. Discussion

By studying the detoxification of 1,8-cineole in the koala we have demonstrated that the koala employs a metabolic strategy which is highly specific and promotes rapid production and elimination of extensively oxidised metabolites. This strategy was similar to that employed for the detoxification of *p*-cymene. The koala excreted seven metabolites of 1,8-cineole and although all seven were quantified, two were present in almost trace amounts. These were 7-hydroxycineole and 7-cineolic acid. Furthermore, the 9-hydroxycineole and 9-cineolic acid each accounted for only 2 - 3 % of the recovered dose. One metabolite, Ci 13, dominated the metabolite profile. When combined with the other hydroxy cineolic acid metabolite, Ci 17, they accounted for 85 % of the recovered dose. The most extensively oxidised metabolite was the dicarboxylic acid metabolite, Ci 22, which accounted for almost 10 % of the recovered dose.

The minor, less oxidised metabolites, underwent extensive conjugation with glucuronic acid. However excretion of conjugated metabolites accounted for only a small percent (~5 %) of the recovered dose.

The specificity of the enzymatic pathways employed to detoxify 1,8-cineole is evident from the metabolite structures (see Table 9.3 and Figure 9.6 for metabolite names and structures). The koala oxidises 1,8-cineole on the C7 and C9 position. This is in contrast to the brushtail possum which oxidised many of the available carbons, including those on the ring structure (Carman and Klika 1992; Bull *et al.* 1993; Carman *et al.* 1994; Carman and Rayner 1994; Carman and Garner 1996). Aliphatic oxidation by the microsomal CYP enzymes is likely to be responsible for the formation of hydroxycineole and dihydroxycineole metabolites, as in the brushtail possum (G. Pass, personal communication). For each metabolite group, the dominant metabolite had undergone the most extensive oxidation at the C9 position (ie. C2, 3 and 13), indicating regioselective preference of oxidative enzymes for this position in the koala.

Further oxidation of the CYP derived metabolites is likely to be catalysed by cytosolic alcohol and aldehyde dehydrogenase enzymes and is responsible for the formation of carboxylic acid metabolites (Alvares and Pratt 1990; Chapter 1 - section 1.5.3). That the koala excreted greater than 85 % of 1,8-cineole as carboxylic acid metabolites suggests that this enzyme pathway has a high capacity and is very efficient. However, investigation into this enzyme system in marsupials is required to confirm its involvement in the detoxification of terpenes.

The absence of significant quantities of precursor metabolites in the urine (ie. the hydroxycineoles and dihydroxycineoles) implies that the oxidative reactions occur rapidly. Rapid production of extensively oxidised metabolites would allow little opportunity for subsequent conjugation and excretion of the less oxidised, precursor metabolites.

Recovery of 1,8-cineole exceeded the estimated amount of 1,8-cineole ingested. The fractions of the dose recovered for koalas 2 and 5 were 1.95 and 1.67, respectively and accounted for the majority of the overall excess recovery. If absorption, metabolism and excretion of ingested 1,8-cineole occurred over a longer period than the 24 h feeding and urine collection, then carryover from the previous days intake

may affect recovery. The amount of leaf ingested by koala 2 was half of that ingested on the previous day. Therefore carryover metabolites from the previous day's intake could contribute to the increased recovery. Similarly, koala 5 excreted minimal urine (26 ml compared to the mean of 101 ml) during the previous day's collection. Therefore, metabolites from the previous days ingestion may have accumulated and been included in the current day's sample. Also, as mentioned previously, accurate intake of 1,8-cineole is almost impossible to measure due to significant variations of terpene composition that can occur within trees. Therefore the variable recovery is attributed to natural variations.

Leaf analysis. E. cephalocarpa is a relatively low oil yield species. However, the yield from the trees used in this experiment were higher than those reported by others. Boland and Brophy (1991) reported a DM total oil yield of 0.7 - 1.8 % of which 85.6 % was 1,8-cineole (other terpene components included limonene 4.8 %, α-pinene 1.6 % and *p*-cymene 0.62 %). 1,8-Cineole was the major component of the terpene profile of leaf, making it the ideal leaf diet for analysing 1,8-cineole metabolism.

Inter-species comparison of metabolism of 1,8-cineole. 1,8-Cineole intakes in the brushtail possum and koala experiments need to be compared before comparisons of detoxification strategies employed by each species can be made. Koalas ingested 2.4 ± 1.1 mmol/kg/day of 1,8-cineole, which was comparable to the calculated possum intake of 2.1 ± 0.5 mmol/kg at 0.5 % (wet weight) 1,8-cineole. However, as discussed in Chapter 10 - section 10.2.3, 1,8-cineole intake was probably overestimated, so an exact comparison of intakes is not possible, although the amount ingested would be comparable to the lower concentrations fed to brushtail possums (ie. 0.5 - 1.0 % wet weight). *E. cephalocarpa* has a low oil content (Boland and Brophy 1991) and presumably the koala would be able to ingest much greater quantities of 1,8-cineole.

The strategy employed by the koala to detoxify 1,8-cineole contrasts with that utilised by brushtail possums. Both species excreted the majority of the recovered dose as hydroxy cineolic acids, however, the brushtail possum excreted eleven different isomers in varying proportions compared to the koala's two, of which Ci 13 accounted for the majority of the recovered dose on its own. The differences in the pattern of oxidation between the two species are easily summarised by grouping metabolites according to the number of oxygen atoms acquired (Figure 11.3). The pattern is similar to that of *p*-cymene (Chapter 8 - Figure 8.2). The koala excreted the majority of the recovered 1,8-cineole dose (> 90 %) as extensively oxidised metabolites (ie. three or more oxygen atoms) compared to 60 % in the possum. In both species, conjugation of the less oxidised metabolites was significant, but a much smaller percentage of the total metabolites was excreted in this form by the koala. In both species of marsupial, it appeared that the less oxidised metabolites were not readily excreted without further alteration to improve their excretability, by either glucuronidation or further oxidation. The brushtail possum utilises the conjugation pathway more extensively than the koala, which prefers the production of extensively oxidised metabolites.

Data from chronic ingestion of 1,8-cineole in the brushtail possum and koala support the observations on detoxification strategies employed in the elimination of *p*-cymene in generalist and specialist folivores.

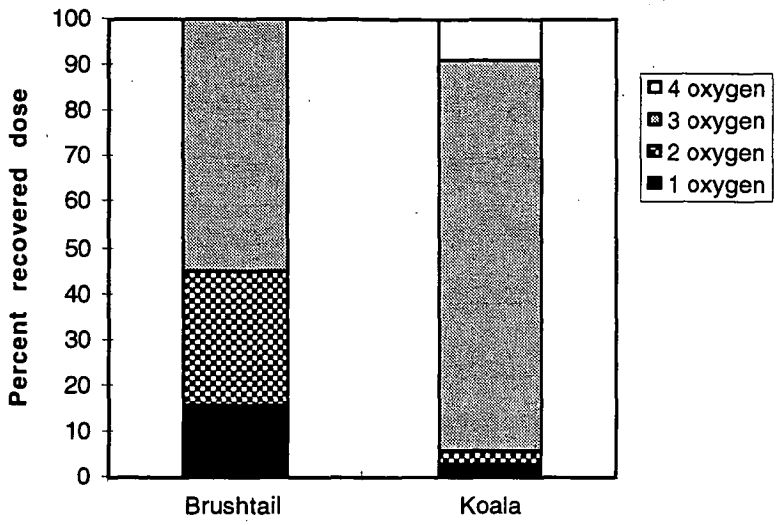

Figure 11.3. Comparative oxidation of 1,8-cineole in the brushtail possum and the koala. Metabolites are categorised by the number of oxygen atoms acquired before conjugation. The values for the brushtail possum are the average of the mean recovery from days 5 and 7 of Experiment 2.

The minor role of glucuronidation of both *p*-cymene and 1,8-cineole metabolites warranted further investigation of koala urine, considering the large amount of glucuronic acid measured. The koala excreted approximately 2 g of glucuronic acid daily and this amount was comparable to that reported by other researchers (Hinks and Bolliger 1956 and 1957; Southwell 1975). Southwell (1975) showed that koalas excreted large quantities of labile glucuronide esters. Since glucuronidation was not significantly involved in the elimination of terpenes, it must have an important role in the elimination of other components of their diet. Glucuronides were identified directly in the urine of koalas using LC/MS and the molecular weights of the aglycones were determined to be those of small phenolic molecules. Baudinette *et al.* (1980) demonstrated that phenol, and its oxidation product quinol, are almost exclusively conjugated to glucuronic acid in both koalas and brushtail possums after intra-peritoneal administration of radiolabelled phenol.

The original source of the phenolic molecules excreted as glucuronides is unclear. The phenolic content of eucalypt leaf is well known, including both hydrolysable and condensed tannins, but relatively little is known about their specific composition (Hillis 1966; Fox and Macauley 1977; McArthur *et al.* 1991; Hume and Esson 1993). The urinary products may originate from either the breakdown of larger phenolic molecules (eg. hydrolysable tannins), or occur naturally in the leaf (eg. gallic acid, catechic and coumaric acid; Hillis 1966). Further investigation into both hydrolysed urine and leaf extracts are required to determine the source of urinary glucuronides.

CHAPTER 12

CONCLUSIONS AND ECOLOGICAL IMPLICATIONS

This research set out to investigate the metabolic fate of two dietary terpenes in marsupial eucalypt folivores. The study was important because it was designed to make comparisons between the metabolism of monoterpenes in all of the four marsupial eucalypt folivores. It has found evidence of adaptations in metabolic pathways with associated dietary specialisation.

The pattern of metabolite excretion for both *p*-cymene and 1,8-cineole varied between the generalist and specialist eucalypt folivores. The specialists demonstrated similar patterns of excretion and were therefore considered as a group.

The oxidative metabolic products of both terpenes were combinations of alcohols and carboxylic acids. Although many metabolites were common to all species, there was a distinct increase in the proportion of more extensively oxidised metabolites in the progression from generalist to specialist folivore. In the case of *p*-cymene, the specialist folivores only excreted metabolites which had undergone oxidation at two or more different sites, whereas the generalist (and the rat) excreted a significant proportion having a single site of oxidation. The pattern was similar with 1,8-cineole metabolism although small amounts of metabolites oxidised at a single site were found in the koala. Furthermore, specialists produced relatively fewer metabolites in total.

The proportion of more extensively oxidised metabolites increased over the first few days of a 1,8-cineole diet. This is evidence that induction of oxidative enzymes occurs.

It was proposed that oxidative enzymes in specialist folivores were more efficient at oxidising many simple monoterpenes. In the generalist, excretion of less oxidised precursor metabolites in either their free or glucuronide form suggests that oxidation occurred more slowly.

Utilisation of the glucuronic acid detoxification pathway was significant in only the rat and the generalist. The fraction of metabolites excreted as glucuronides increased with time when 1,8-cineole was introduced into the diet, indicating induction of glucuronyl transferases which supports similar evidence of glucuronyl transferase induction in ringtail possums (McLean *et al.* 1993). Although minor amounts of terpene glucuronides may be excreted in specialists with chronic ingestion, this pathway was not used extensively. This was a major difference in the metabolic strategies employed by the generalist and specialist folivores.

Historically, glucuronuria was attributed to the elimination of essential oils in folivorous maruspials. We have demonstrated that glucuronidation is important in the elimination of these compounds in the brushtail possum but not in the specialist folivores.

We can only postulate on the reasons why specialists use a different strategy in the elimination of simple monoterpenes compared to the generalist. Given that simple

monoterpenes are always present in the eucalypt leaf diet of the folivores, differences would indicate an adaptation of detoxification mechanisms to diet. An enhanced capacity of liver, and possibly other metabolically active tissues, to extensively oxidise compounds such as terpenes in specialists would support this idea.

It is proposed that the different strategies employed are based on factors such as a balance of dietary energetics or enzyme availability. As the eucalypt component of the diet increases, the diet becomes nutritionally limited, as it contains low levels of nitrogen as well as being high in PSMs and fibre (Cork 1984; Foley 1987). Therefore, metabolic costs of metabolising such compounds could potentially become a significant factor in the metabolic pathways employed.

The findings reported in this thesis do not directly support the widely regarded hypothesis that herbivores tend to be generalist feeders as ingestion of smaller quantities of a range of PSMs avoids saturation of enzymatic detoxification pathways (Freeland and Janzen 1974). The research in this thesis demonstrates metabolic adaptations which result in successful specialisation on a diet often containing large amounts of potentially toxic compounds. Furthermore, it has been demonstrated that brushtail possums have a large and non-saturable (up to 4 % wet weight of 1,8-cineole) capacity for detoxifying a single PSM, 1,8-cineole, while feeding on an otherwise favourable diet.

The issue of diet selection is obviously very complex. It is becoming apparent that multiple factors influence diet choice. Such factors include quality of diet as well as PSM profile and load. The Freeland and Winter (1975) hypothesis can probably be reinterpreted as a result of a complex interrelationship between a nutritionally poor diet plus a detoxification challenge from a mixture of PSMs. It must be remembered that detoxification conditions were optimal in this study due to a balanced diet, therefore competition for enzymes or metabolic substrates such as glucuronic acid were minimal.

The toxic potential of simple monoterpenes in marsupial eucalypt folivores may need to be reconsidered. Although highly toxic in other animal species, the folivores have evolved effective and high capacity mechanisms for detoxifying 1,8-cineole, *p*-cymene and presumably many other terpenes. Their ability to do so seems to render these terpenes essentially non-toxic in these species.

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Appendix 1: Preliminary Pharmacokinetic Investigations of 1,8-Cineole in Brushtail Possums

A1.1. Introduction

Method development and preliminary, but incomplete, data on the the blood concentration time profile of 1,8-cineole in the brushtail possum is reported in this appendix. The findings to date suggest a significant new dimension in our understanding of the role of 1,8-cineole, as a PSM, in diet selection in this generalist herbivore.

There exists a deficit of pharmacokinetic studies of PSMs in herbivores. Yet, combined with an understanding of the metabolic fate and detoxification pathways of PSMs, pharmacokinetic data could provide strong evidence for refining hypotheses on diet selection in herbivores, which in the past have been largely based upon observational data alone.

As there was little technical information available in the literature concerning techniques for sampling and measuring blood in brushtail possums (Duckworth and Meikle 1995; Eason *et al.* 1999), novel methods were developed.

The first method described in this chapter measured levels of 1,8-cineole in expired air. In accordance with current ethical standards for animal use in experimental research, sampling expired air subjected possums to minimal stress and discomfort. The use of breath analysis as an indirect measure of blood concentrations is well established. The physiochemical properties of 1,8-cineole make it ideal for analysis by this method.

A number of human studies have detected unchanged monoterpenes in expired air after inhalation exposure. Gerarde (1960) reported that a small fraction of *p*-cymene was excreted in expired air. Approximately 8 % of inhaled α-pinene is also eliminated unchanged in exhaled air (Falk *et al.* 1990; Levin *et al.* 1992).

In the method developed in this study, expired air was collected onto charcoal traps and desorbed using an organic solvent prior to analysis by GC.

Results from expired air sampling produced a recurring double-peak phenomenon in the expired air concentration-time profiles of 1,8-cineole. The need to verify the results justified the development of a method for analysing 1,8-cineole directly in the blood.

Brushtail possums are wild animals and can be difficult to handle if provoked. Considerable experience was gained in developing a technique for taking frequent, serial blood samples over an extended time period (6 hours). On a number of occasions the experiment was aborted before the optimal sampling period was reached, due to problems with maintaining sedation for extended periods. For this reason a satisfactory 1,8-cineole time profile was acquired from only one possum.

The method used to analyse 1,8-cineole blood levels required development. Solid phase microextraction (SPME) was ideally suited for the purpose of measuring volatile 1,8-cineole in the headspace above blood samples. This relatively new sample preparation technique had the advantage of being fast, required no organic solvents, had low detection limits and its versatility allowed it to be be useful in this application.

SPME concentrates volatile and non-volatile organic compounds from either liquid or solid samples for analysis by GC, GC/MS or HPLC. A SPME unit (Supelco, Bellefonte, Pasadena, USA) consists of a length of fused silica fiber coated with a polymer (polydimethylsiloxane, PDMS). The fibre is protected by a needle attached to a plunger. When the plunger is depressed, the fibre is exposed to either the headspace of the sample or directly into a liquid sample. Analytes adsorb onto the fibre (only volatile compounds are adsorbed from the headspace) during the sampling and adsorption period. The fibre is then introduced into the injector port of either the GC or HPLC and analytes desorbed by temperature or solvent, respectively.

The successful 1,8-cineole blood analysis partially validated the double-peak phenomenon observed in the expired air sampling. Although multi-peak phenomena have been described for a number of diverse compounds (Veng-Pedersen 1980; Plusquellec *et al.* 1987; Piquette-Miller and Jamali 1997; Wang *et al.* 1999), the hypothesised mechanism in each case is speculative. The 1,8-cineole double-peak phenomenon, which is excessively delayed compared to those examples previously reported in the literature, may affect diet selection in possums.

Approval for this study was obtained from the Ethics Committee (Animal Experimentation), University of Tasmania (Approval # 97015) and Wildlife Service, Department of Primary Industry, Water and Environment.

A1.2. Methods

A1.2.1. Expired air sampling

A1.2.1.1. Possum experimentation

Six brushtail possums (2 females and 4 males; identified as BP 7 - 12) were trapped from the grounds of the University of Tasmania. They were individually housed in purpose built, covered, outdoor enclosures and provided with a nest box and aerial branches. Possums were maintained on a diet of chopped apples, carrots, lettuce leaf and occasionally bread, dog biscuits and bananas for variety. Possums were dosed intragastrically with 400 mg/kg of 1,8-cineole using a flexible Gentle-Feed paediatric feeeding tube (8.0Fg x 38 cm, Mallinckrodt Medical, Dublin, Ireland) as described in Chapter 4 - section 4.2.1.

The 1,8-cineole dose was chosen with two considerations in mind. First, the dose had to be tolerated by fasting animals when ingested as a bolus. Our experience with *p*-cymene bolus dosing (200 mg/kg *p*-cymene was well tolerated with no apparent

adverse effects) combined with the knowledge of amounts of 1,8-cineole consumed during 1,8-cineole feeding experiments (maximum 1,8-cineole intakes were approximately 12 g/day; Chapter 10) indicated that the chosen dose would be tolerated safely. Second, the dose had to be as large as possible to assist the detection of 1,8-cineole after ingestion and first pass metabolism.

The 1,8-cineole was administered quickly by oral gavage and possums were released into a purpose-made 15 litre glass expiration chamber held securely on a wooden frame (Figure A1.1). A wire mesh floor suspended over the bottom of the glass chamber assisted in keeping possums dry and comfortable throughout the experiment. The chamber was covered and darkened with a blanket and possums returned to sleep within a short period of time. At the end of the experiment, they were coaxed out of the chamber with banana and by blowing air onto their backs, which they disliked. This sampling method caused minimal stress for the possums.

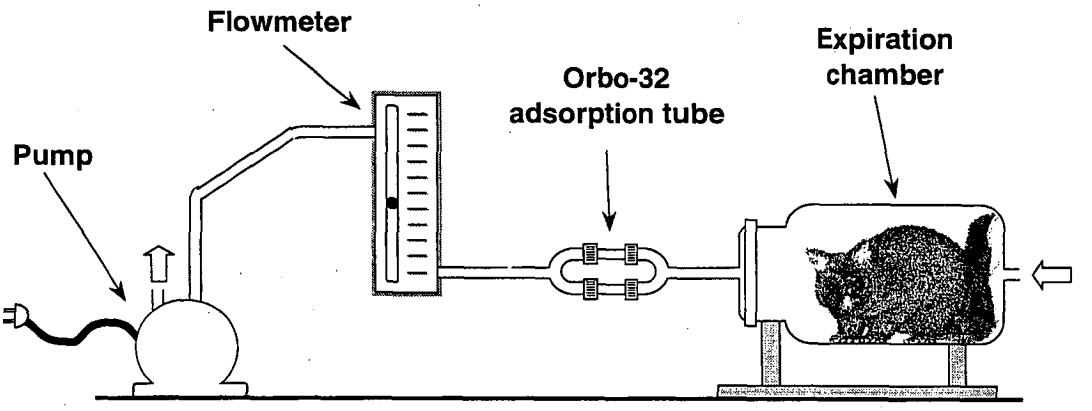


Figure A1.1. Schematic of the apparatus used for sampling expired air in brushtail possums. Possums were placed into the darkened glass chamber after being administered 1,8-cineole, orally. The chamber was sealed and connected to a pump (4.5 l/min) which drew air in from the chamber through two parallel Orbo-32 absorption tubes and a flow meter. Expired air was sampled for 5 or 10 min at regular intervals. In between samples, the pump was connected directly to the chamber.

Air flow through the system was based on the recommendation that an adult possum would require a minimum of 0.5 ml of oxygen/g body weight/h, when resting at thermoneutrality (Dr Stewart Nicol, Department of Anatomy and Physiology, University of Tasmania, personal communications). Therefore, to maintain oxygen levels around 20 %, a minimal flow rate of 4 l/min was required.

The front of the chamber was sealed with an outlet cover. A continual flow of air was drawn through the chamber at 4.5 l/min by an Air-Cadet (model 7530) electric pump (Cole-Parmer Instrument Company, Vernon Hills, Illinois, USA). Air inlets were positioned on the rear wall and the rear floor (urine drainage port) of the chamber. Two freshly opened Orbo-32 adsorption tubes containing activated

coconut charcoal (Supelco Inc, Bellefonte, Passadeena, USA; see Figure 12.2) and a flow meter were placed between the chamber outlet and the pump, respectively. All post-chamber components were connected with glass tubing, joined by minimum lengths of plastic tubing.

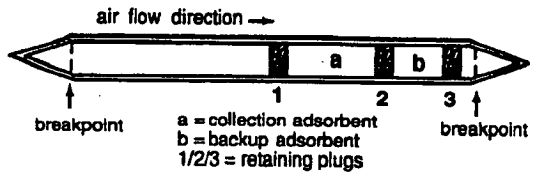


Figure A1.2. Schematic of the Orbo-32 adsorption tube used for sampling 1,8-cineole in expired air of brushtail possums. The tube has two adsorbant beds of activated coconut charcoal (a and b). The second smaller bed is used to detect whether excessive breakthrough occurs. The air flow through the tube is indicated by the arrow. (Reproduced from the ORBO tube instruction sheet, Supelco, Bellefonte, Pasadena, USA).

Outlet air was sampled for 10 or 15 min periods at timed intervals of between 15 and 30 min for up to 6 h (early samples were collected continuously). Fresh Orbo - 32 absorption tubes were placed in parallel at the start of each sampling period. In between samples, the pump was connected directly to the chamber outlet, to ensure consistent airflow throughout the experiment.

A1.2.1.2. 1,8-Cineole analysis

The contents of both charcoal tubes from each sample period were combined in a miniature glass vial and desorbed. *p*-Cymene (0.25 mg/ml) was the internal standard and was dissolved in solvent, carbon disulphide (CS_2), and 1 ml added to each vial. The vial was capped and allowed to sit at room temperature for 30 min before analysis by GC/FID.

Analyses used the Varian 3300 GC and capillary column described in Chapter 2-section 2.2.1. GC conditions were: injector 250°C, split ratio 1:10, carrier He (9 psi), oven isothermal at 100°C and detector 300°C, injection volume 1 µl.

Orbo-32 adsorption tubes are manufactured for analysing air quality and detecting contaminants (Supelco product manual, 1998). Their use in the system described here was unconventional and therefore required validation. The maximum recommended airflow through Orbo-32 Tubes is 1000 ml/min resulting in a combined maximum airflow across the parallel tubes of 2000 ml/min. This flow was necessarily exceeded to ensure oxygen concentrations were maintained for the possums. The performance of the tubes at this flow rate had to be determined.

The tubes are divided into two adsorbent beds. The first adsorbent bed is designed to trap the compounds of interest, while the second smaller bed is a backup section to determine whether breakthrough from the front compartment has occurred. If the backup compartment contains > 10 % of the recovered contaminant then the tube is considered to be overloaded.

A sample pair of tubes from an expired air experiment were tested for breakthrough. The charcoal from the front and back compartments from each of the vials were treated separately. The breakthrough compartments in each tube contained 2.9 and 1.3 % of the front compartment, respectively. In each case breakthrough was within acceptable limits.

A1.2.2. Blood sampling

The 1,8-cineole versus time data from expired air experiments required validation due to the second, delayed, 1,8-cineole peak observed in all experiments where sampling was persisted with for adequate time. Extracting serial blood samples from brushtail possums proved difficult. The frequency of sampling precluded the use of multiple jugular venepuncture as described in some studies (Viggers and Lindenmayer 1995; Eason *et al.* 1999). Cannulation of the cephalic vein on the ventral surface of the forelimb proved to be the best site. However, difficulties arose from a combination of keeping the cannula viable for the duration of the experiment, maintaining a suitable level of sedation throughout and the process of gaining experience in these techniques. Furthermore, the second 1,8-cineole peak occured at variable times requiring frequent sampling throughout.

A1.2.2.1. Possum experimentation

Anaesthesia/sedation. The dissociative anaesthetic Zoletil 50 (Virbac (Australia) Pty Ltd, Peakhurst, NSW) was the agent used for sedation. Zoletil contains equal proportions of tiletamine, a cyclohexamine derivative which produces dissociative anaesthesia, and zolazepam, a benzodiazepine which causes sedation and muscle relaxation. Dosage regimes for sustained sedation were determined experimentally. Fasted possums were initially sedated with an IM loading dose of Zoletil 10 mg/kg into the gluteal muscles. This dose was based on suggestions in the Zoletil product manual and literature references for *T. vulpecula* and *T. caninus* (mountain brushtail possum) (Duckworth and Meikle 1995; Viggers and Lindenmayer 1995). Sedation occurred within 2 - 4 mins. One possum (BP 8) required a second IM dose of 3.5 mg/kg after 4 mins as he was still awake.

Sedation was sustained by giving additional doses of 1/4 to 1/3 of the loading dose via the iv cannula (Viggers and Lindenmayer 1995). The cannula was flushed with heparinised saline after each additional dose. We found the level of sedation and time of recovery varied between possums and was influenced by the amount of movement the possum attempted. Once the cannula was in place, quite a low level of anaesthesia or sedation was sufficient. Top-up doses of Zoletil were only administered once the possum was able to withdraw its forelimb from the painless procedure of drawing blood from the cannula. At this stage possums were able to make gross movements and were carefully protected from injury. Movement often

induced further sedation, presumably due to redistribution of the anaesthetic agent with increased muscle blood flow associated with the movement. We considered that it was beneficial to the possums well being to be allowed to move throughout the extended duration (up to six hours) of the experiments.

Observations were made on each possum throughout the experiments. Respiration rate, response to pinching extended paw and brushing whiskers were monitored to gauge the level of sedation at each blood sample. Other signs such as extent of mobility, hypersalivation, lip licking and degree and extent of muscle tone were also noted.

Possums were allowed to recover from the anaesthesia in a padded metabolism cage overnight (temperature maintained at 20°C). Normal diet and water were supplied *ad lib.*

Cannulation. A 22 G flexible cannula (Optiva I.V. Catheter, 22G, 25 mm long, ID 0.6 mm, OD 0.9 mm, sterile radio opaque polyurethane teflon coated; Johnson & Johnson, Australia) was used. Initially we attempted to locate and cannulate a lateral tail vein, recommended by Duckworth and Meikle (1995). This proved to be a very poor site as the vein was difficult to detect and too small to reliably insert the cannula. The cephalic vein on the ventral forelimb was well defined with proximal occlusion of the vein and proved to be a superior site for cannulation.

The site was shaved and the cannula taped into position with Leukosilk tape (Beiersdorf, Hamburg, Germany). Betadine (povidone iodine solution 10 % w/v, Faulding Pharmaceuticals, Salisbury, South Australia) was used to sterilise the wound once the cannula was removed.

1,8-Cineole dosing. A mixture of 1,8-cineole (500 mg/ml) in peanut oil was prepared. Once the posssum was sedated and the cannula inserted it was dosed orally with 1,8-cineole, 400 mg/kg, as described in A1.2.1.1.

Blood sampling. Blood samples were collected at regular intervals throughout experiments. The cannula was maintained by flushing with heparinised saline (50 IU in 5 ml; Astra Pharmaceuticals Pty Ltd, North Ryde, NSW) after each blood sample was removed. Approximately 50 µl of blood/heparinised saline was drawn from the cannula and discarded prior to the blood sample being collected. This was to ensure that the actual blood sample was undiluted. A 1 ml luer lock syringe was used for drawing the blood and between samples the cannula was capped with a rubber bung.

The blood was placed into prepared heparinised vials for storage. The vials were two ml screw cap septum sealed autosampler vials, placed in a rack and 100 µl of heparin sodium (porcine mucous) 1000 IU/ml (Heparin Injection B.P., David Bull Laboritories) measured into each vial. The heparin was washed around the walls of the vial and then allowed to dry overnight at 80°C in an oven.

The blood samples were placed immediately into a metal block sitting in ice until they were transferred to a refrigerator (4°C).

A1.2.2.2. SPME assay

At the time of the development of this method there were few reports on the use of SPME in analysing blood or for quantitative analyses. A considerable amount of 'trial and error' was required before the sampling method was reproducible.

Instruments and conditions. The GC/MS described in Chapter 2 - section 2.2.1 was used for SPME desorption and analysis.

A Hewlett-Packard HP-1, 25 m x 0.32 mm ID GC capillary column, lined with 0.25 µm crosslinked 1 % phenyl methyl silicone, was used for chromatography. The GC/MS operating conditions were as follows: injector 220°C, splitless for 2 min then purged, oven 30°C for 2 min (desorption period) then 25°C/min to 175°C, carrier gas He at a pressure of 15 psi. Although the TIC chromatogram contained only 1,8-cineole and internal standard peaks, selected ion monitoring (SIM) scan mode was used to ensure good resolution of 1,8-cineole and *p*-cymene. The seleceted ions used were at *m/z*'s 134 and 119 for 1,8-cineole and 154 and 139 for *p*-cymene. The mass spectrometer conditions were: EM voltage of 200, scan every 50 ms and mass range of 40 - 450.

SPME conditions. The SPME holder was used with a 100 µm PDMS fiber. Sealed vials were allowed to equilibrate to room temperature (22°C) and pressure for a minimum of 30 min before SPME analysis. An improvised sampling apparatus was devised to shake the sample during adsorption. Vials were placed in a holder attached to a gently vibrating vortex mixer. The SPME unit was held securely in place by a clamp attached to a retort stand. The septum was punctured by the filament needle and the fibre exposed to the headspace for 7 min. The fibre was then retracted and the SPME unit removed and introduced into the GC injector immediately. The SPME fibre was conditioned before each period of use by inserting it into the GC injector (220°C) for 5 min.

Optimal SPME conditions were determined using blank possum blood spiked with a stock solution of 1,8-cineole. A primary solution of 50 mg 1,8-cineole in 10 ml methanol was prepared. A secondary aqueous stock solution was prepared by measuring 50 µl primary solution and diluting to 50 ml with water. The final 1,8-cineole concentration was 0.25 µg/50µl. Fifty µl of blood was meaured into a 4 ml septum sealed screw cap miniature glass vial plus 50 µl aqueous terpene stock solution.

Determining optimal adsorption time. The optimal adsorption time was determined by sampling replicate vials for variable times (1 to 10 min). The absolute counts of 1,8-cineole were plotted and the maximal absorption determined at the minimum time (Figure A1.3). An adsorption period of 7 minutes was decided upon to ensure that the sample was at equilibrium.

Reproducibility of SPME sampling. The SPME method depletes the sample significantly and multiple sampling of a single vial results in a logarithmic rate of depletion (Figure A1.4). Sample depletion was measured to be 84 ± 5 % (mean ± sd) of the remaining 1,8-cineole after each sampling. Although not a conventional method of testing repeatability, the R^2 value of the resulting logarithmic plot of 1,8-cineole

abundance versus number of times the vial was sampled provided an indication of the reproducibility of the sampling and analysis technique. The results from a single vial sampled six times is shown in Figure A1.4.

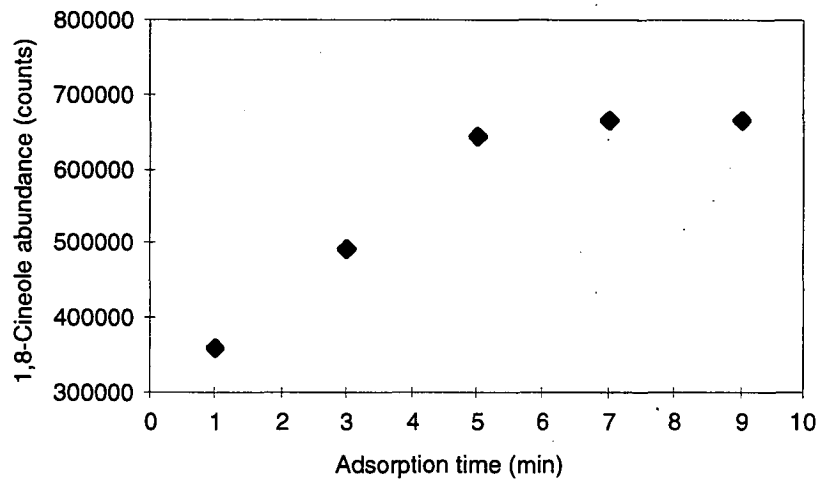


Figure A1.3. The optimal adsorption time for 1,8-cineole, spiked into blood, onto the SPME fibre occurred between 5 and 7 min. Seven minutes was chosen for the absorption period to ensure equilibrium was reached in all samples. Data plotted here are from a typical analysis using variable adsorption times (1, 3, 5, 7 and 9 min) and the abundance of 1,8-cineole by GC/MS analysis (conditions described in Section A1.2.2.2).

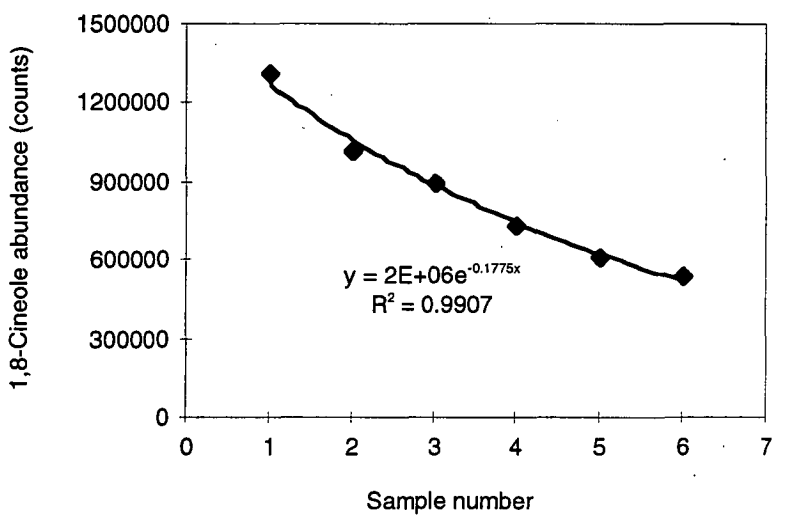


Figure A1.4. Serial sampling from one vial shows a constant rate of sample depletion. Each sampling removed 84 ± 5 % (mean ± sd) of the remaining sample. The R^2 value provides an indicator on the repeatability of the sampling procedure.

Replicate vials were also sampled for within day and between day repeatability of the sampling method. Ten vials of blank possum blood (50 µl) were spiked with 50 µl of the aqueous 1,8-cineole stock solution. The coefficient of variation (CoV) for 1,8-cineole abundances in five replicates each for within day and between day reproducibilities were 3.2 and 7.1 %, respectively and were within acceptable limits.

Attempts to identify an appropriate internal standard. Attempts were made to find an appropriate internal standard to minimise error in the quantitation of 1,8-cineole blood levels. Both *p*-cymene and dihydro-α-terpineol were trialed. We found that SPME resulted in a complex system in which small differences in physicochemical properties (eg. boiling point, polarity, molecular weight, bond structures etc) resulted in significant differences in partitioning presumably between both the liquid-headspace and headspace-fibre phases. Figure A1.5 shows the different rates of depletion between 1,8-cineole and *p*-cymene and 1,8-cineole and dihydro-α-terpineol when a single vial was sampled repeatedly. Although the preparation of calibration curves over appropriate concentration ranges would account for such differences, it was decided that using absolute abundances of 1,8-cineole was satisfactory, given the acceptable repeatability of the analysis.

A1.3. Results

A1.3.1. Expired air sampling

Results from expired air experiments are shown in Figure A1.6 for four possums in which air was sampled for sufficient time and frequency to show the two distinct peaks in expired 1,8-cineole levels (ie. the double-peak phenomenom). The observation was unexpected, however, its recurring nature suggests the measurements are real and reproducible. The second peak occurred between four and six hours after the 1,8-cineole dose in each possum, however, the relative sizes of the first and second peaks were variable.

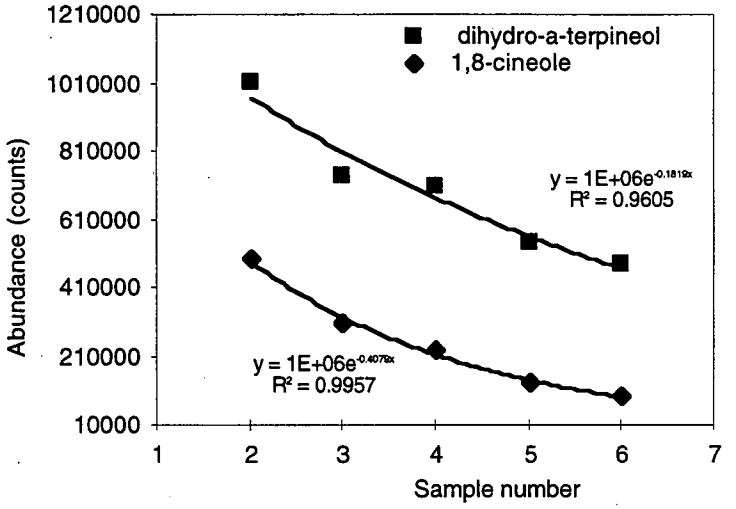
An absorption phase was evident in only one possum (BP 10). However, the first elimination phase allowed an estimation of the half life ($t_{1/2}$) of 1,8-cineole in each possum. Half lives ranged from 16 - 31 mins. Sufficient data points were obtained from three of the possums to characterise the elimination of 1,8-cineole after the second peak and values ranged from 15 - 49 min. There was good agreement in the $t_{1/2}$ calculated for the first and second peaks for each possum (Anova (two factor with replication) for n = 3 possums $df = 1$, $F = 0.88$, $P = 0.40$).

A1.3.2. Blood sampling

Headspace SPME of 1,8-cineole blood resulted in a gas chromatogram which contained only the 1,8-cineole peak (and internal standard peaks when trialed).

A comprehensive set of data from blood samples covering the time period required to observe the second peak were collected successfully from one possum only

A) 1,8-cineole and dihyro-α-terpineol in water



B) 1,8-cineole and *p* -cymene in water

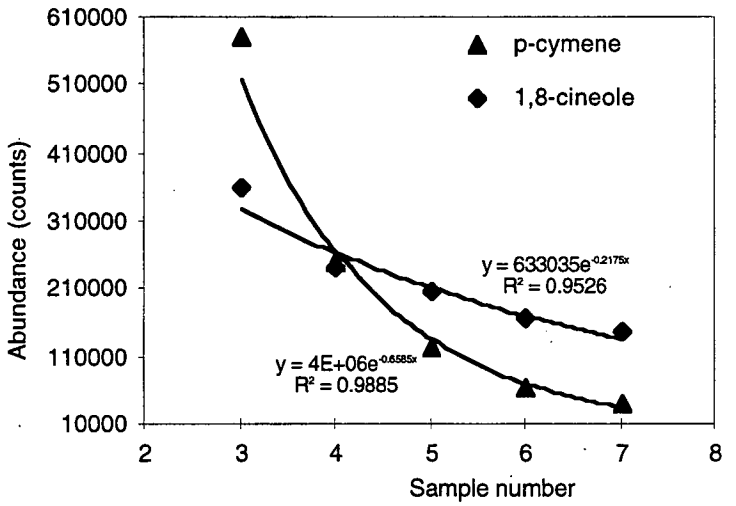


Figure A1.5. Comparisons of the rates of depletion during serial sampling of a single vial, between 1,8-cineole and two trial internal standards: A) *p* -cymene and B) dihydro-α-terpineol. Differences in their respective physicochemical properties results in different partitioning coeeficients between the liquid-headspace and the headspace-fibre. In both examples, the first one or two samples analysed overloaded the GC/MS capillary column and therefore results are meaningless.

(Figure A1.7). The 1,8-cineole time profile reflected an initial absorption phase, with peak blood levels occurring at approximately 30 min. The absorption phase was followed by an elimination phase with a half life of 28 min. The second delayed rise in 1,8-cineole blood levels was evident, although blood sampling was discontinued before elimination phase samples were collected. Thus the 1,8-cineole-time profile of blood reflected the same pattern observed in expired air. Further experiments are required to enable the double-peak phenomenom to be described and characterised fully.

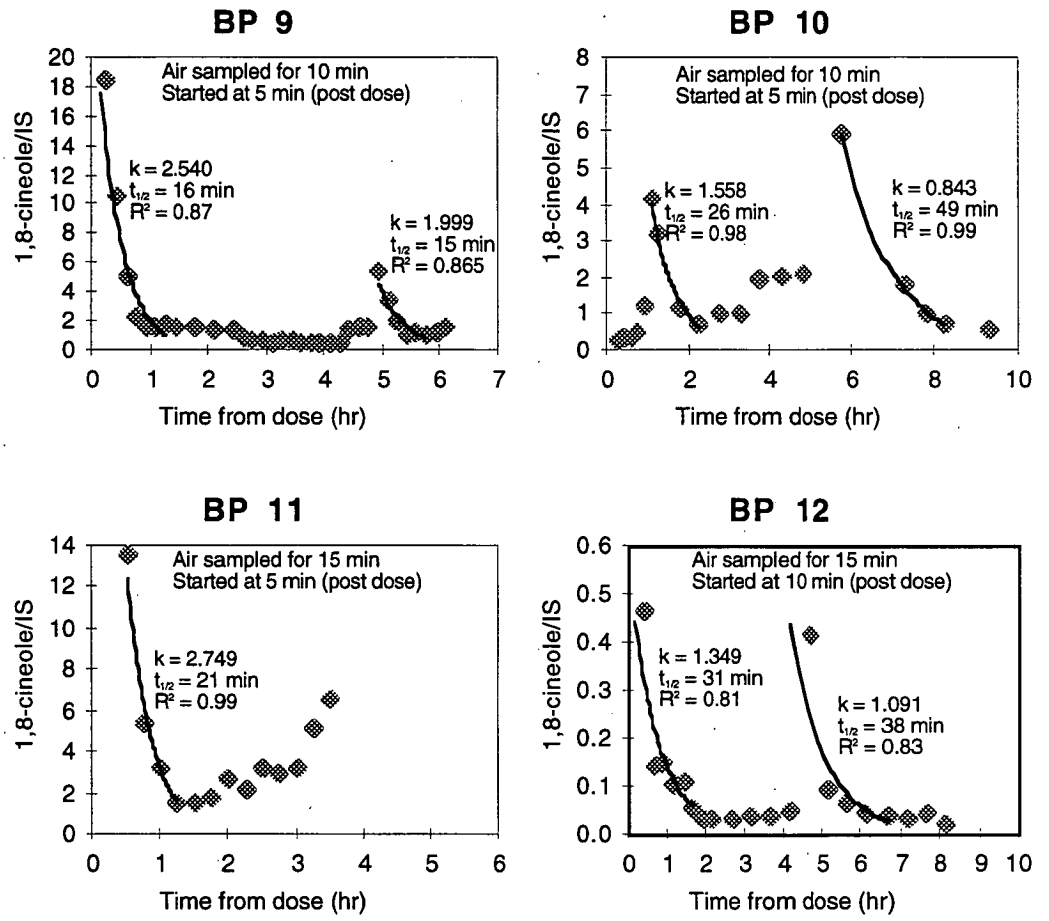


Figure A1.6. Expired air 1,8-cineole/internal standard (IS) time profiles in brushtail possums (BP 9, 10, 11 and 12), each gavaged with a single oral dose of 1,8-cineole (400 mg/kg). Exponential regression lines for both the first and second (where appropriate) elimination phases for each possum were calculated (marked on individual graphs). The k-value (-slope), $t_{1/2}$ ($t_{1/2}$=0.693/k) and R^2 of the regression line for each elimination phase are reported.The scale of 1,8-cineole/IS for BP 12 is an order of magnitutde less than that of the other possums, no explanation for this difference was found.

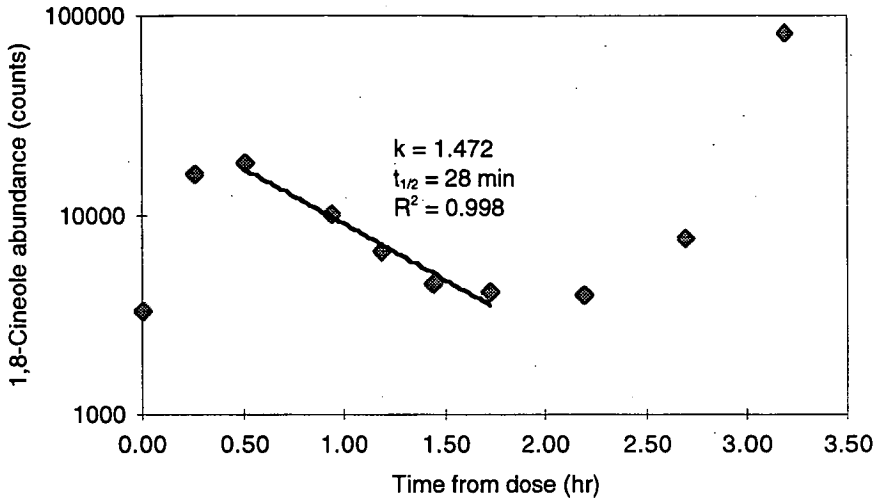


Figure A1.7. Blood 1,8-cineole time profile after an oral dose of 1,8-cineole (400 mg/kg). An exponential regression line for the first elimination phase is marked. The k-value (-slope), $t_{1/2}$ ($t_{1/2}$=0.693/k) and R^2 for the regression line are also reported. The blood levels appear to confirm the double-peak phenomenum of 1,8-cineole blood levels, although the elimination phase of the second peak was not collected.

A1.4. Discussion

To date there has been a paucity of pharmacokinetic data in marsupials, particularly regarding PSMs in herbivores. Incomplete but novel and potentially significant findings have emerged from the preliminary results presented here. Now that analytical methods are established, further possum dosing and sampling experiments are necessary to characterise and explain the processes involved in the double-peak phenomenon as well as the ecological significance of the observation.

The pharmacokinetics of 1,8-cineole have been reported in humans after inhalation exposure (Stimpfl *et al.* 1995; Jager *et al.* 1996). Elimination from blood in humans has been shown to be biphasic, with a short distribution half life (~10 min) and a long elimination half-life (~105 min; Stimpfl *et al.* 1995; Jager *et al.* 1996). The limited pharmacokinetic data acquired from possums to date indicate that 1,8-cineole is rapidly absorbed after an oral dose with maximum concentrations generally occurring within 30 min. The second peak confuses the 1,8-cineole-time profile making it difficult to determine whether elimination was biphasic in possums. At this stage it is unclear whether the first halflives (15 - 50 min), measured immediately after absorption, were reflecting distribution or elimination. However, it would seem likely that the physicochemical properties of terpenes would predispose them to being distributed into fatty tissue in all animal species. After the initial elimination phase, blood levels of 1,8-cineole remained elevated. Whether this reflects biphasic elimination or is a result of disruption of gastric function and a trickle through of 1,8-cineole is speculative and requires further investigation.

From the results reported in this appendix, there appears to be considerable variation in the relative size of the second peak compared to the first as well as the delay in the second peak.

Initial results suggest that measuring the expired air concentrations of volatile compounds has good potential as a non-invasive method of collecting pharmacokinetic data in difficult to handle animals. In this case, possums were able to continue their normal daytime sleep once the dose had been administered. Maintaining experimental animals in an unstressed, as close to normal condition, increases confidence in results. In comparison, blood sampling required possums to be sedated for a number of hours, introducing unknown factors such as effects from the sedative, position of the possum (possums were maintained in a supine or lateral position throughout, possible affecting gastric emptying) and stress associated with recovery from anaesthesia/sedation.

Future directions. The double-peak phenomenon observed in this preliminary work requires further investigation to understand the mechanisms involved and what implications the phenomenon may have for digestion and diet selection. Now that the analytical methods of analysing 1,8-cineole concentrations in both expired air and blood samples are established, further experimental work will provide valuable data.

The cause of the second peak can only be speculated upon at the present time. This phenomenon has been attributed to many possible causes in the diverse range of compounds reported to have multiple-peak plasma concentrations. Fluctuations in gastric emptying is thought to be responsible for the double-peak phenomenon of cimetidine (Oberle and Amidon 1987). It is unlikely that normal fluctuations in gastric emptying account for the double-peak of 1,8-cineole in possums since the second peak occurred after a delay of four to six hours in each case. Oral administration of the benzodiazepine, alprazolam, in rats resulted in the double-peak phenomenon and the underlying mechanism was attributed to a reduction in gastric motility caused by the muscle relaxant effect of alprazolam (Wang *et al.* 1999). 1,8-Cineole may also exert a similar physiological effect on gastric function, initiated by elevated blood concentrations of 1,8-cineole. Pharmacological effects of 1,8-cineole include CNS depression (Jenner *et al.* 1964; Patel and Wiggins 1980) potentially affecting gastric function. Administering 1,8-cineole intraperitoneally (ip) or intravenously (iv) would assist in determining whether the phenomenon observed in possums is associated with a disrupted absorption pattern. Interestingly, if this were the case, the trigger to re-establish gastric function is not activated by a minimum threshold blood concentrations of 1,8-cineole, as the second peak in blood level is excessively delayed.

Further modifications in the blood sampling technique may improve the procedure. As it stands, the method is stressful for the possums, with prolonged sedation being undesirable. Future experiments will attempt to maintain possums in a more normal state. Ideally, possums will be sedated only for the purpose of inserting the cannula. It is envisaged that a cannula extension, threaded through the fur to a remote location on the possums back or the insertion of a vascular access port will allow the possum to resume its normal daytime sleep throughout the sampling regime (Cleva *et al.* 1995).

Appendix 2: Supporting data for *p*-cymene metabolism in rats

A2.1 Introduction

The following tables support the data reported for *p*-cymene metabolism in rats presented in Chapter 3.

Table A2.1. Calibration curve concentrations and parameters for metabolites quantified in the rat.

		Cy 1	Cy 2	Cy 5	Cy 6
Stock soln conc (µmol/400µl methanol)		6.5	7.5	6.8	7.9
Dilution #	Stock soln[1] µl			µmol/ml	
1	0	0.00	0.00	0.00	0.00
2	10	0.16	0.19	0.17	0.20
3	20	0.32	0.38	0.34	0.40
4	30	0.48	0.57	0.51	0.60
5	40	0.64	0.76	0.68	0.80
6	50	0.80	0.95	0.85	1.00
n (calibration curves)		6	6	6	6
Equations (mean)		y=1.070x-0.001	y=1.371x - 0.030	y= 1.376x - 0.078	y= 1.463x - 0.026
R^2 (mean)		0.999	0.975	0.982	0.986

[1]Stock solutions were diluted to 1 ml with blank urine.

Table A2.2. Individual rat details including sex, weight, dose and absolute molar recovery of each metabolite, excreted in 48 h, for each dose.

GG	Sex	Weight	Dose	Metabolite recovery µmol					
		g	µmol	Cy 1	Cy 2	Cy 5	Cy 6	Cy 8	Total
A) Dose 0.37 mmol/kg									
Free									
1	m	310	128	10.3	8.8	4.3	5.5	0.0	28.9
2	m	310	128	5.9	11.0	6.4	8.0	0.0	31.3
5	f	245	96	9.6	6.6	2.5	2.6	0.0	21.3
6	f	270	104	3.9	3.5	2.6	2.6	0.0	12.5
7	f	190	71	17.7	14.4	7.8	7.1	0.0	47.0
8	f	225	83	7.2	11.7	12.2	8.6	0.0	39.6
Total									
1				38.3	17.7	9.9	13.1	15.6	94.6
2				30.8	16.3	5.3	9.1	12.3	73.8
5				26.9	12.3	7.5	8.9	12.6	68.2
6				30.8	11.8	8.8	10.8	15.6	77.6
7				20.0	10.4	4.1	5.6	7.8	47.8
8				12.4	6.3	5.4	6.8	4.6	35.5
B) Dose 1.49 mmol/kg									
Free									
3	m	390	582	-	-	-	-	-	-
4	m	360	537	77.8	66.2	30.4	30.2	4.1	208.7
5	f	240	358	57.2	46.1	14.6	13.5	10.9	142.2
6	f	270	403	36.6	34.8	14.6	18.2	3.6	107.8
7	f	210	313	53.2	42.4	18.7	18.2	0.0	132.5
8	f	240	358	57.3	53.2	24.5	30.5	3.4	168.9
Total									
3				85.1	68.1	37.8	40.0	66.7	297.7
4				111.0	76.0	32.7	41.6	48.8	310.2
5				76.3	44.6	16.3	22.2	36.0	195.4
6				56.5	34.8	15.8	22.5	31.8	161.5
7				66.7	44.6	14.4	26.2	44.9	196.8
8				59.7	34.0	18.5	23.8	22.3	158.4

Table A3.3. Reconciling the urinary measurements of conjugated metabolites (total - free metabolite) with glucuronic acid (GA) after each doses of *p* -cymene in the rat.

Rat	Day 0[1]		Day 1		Day 2	
	Conjugated metabolites	Urinary GA	Conjugated metabolites	Urinary GA	Conjugated metabolites	Urinary GA
	µmol/kg					
Dose 0.37 mmol/kg						
1	0	282	176	-	0	113
2	0	119	104	-	0	102
5	0	45	139	268	14	214
6	0	135	184	452	17	230
7	0	194	0	158	0	68
8	0	181	0	199	0	134
Mean	0	159	101	269	5	144
sd	0	80	83	130	8	65
P -value[2]				0.15		0.35
Dose 1.49 mmol/kg						
3	0	131	-	495	24	203
4	0	154	163	767	1	236
5	0	146	140	759	0	277
6	0	197	154	604	0	452
7	0	266	181	767	0	297
8	0	217	0	659	0	217
Mean	0	185	127	675	4	280
sd	0	51	73	111	10	92
P -value[2]				0.001		0.06

[1]day 0 is the pre-dose control .

[2]Student's paired *t* -test comparing conjugated metabolites with GA (minus pre-dose GA).

"-"urine was unavailable for measurements.

nb. 1.0 µmole glucuronic acid = 194 µg

Table A2.4. Urine and faeces output for individual rats during *p* -cymene dosing experiments.

A) Dose = 0.37 mmol/kg

	Day 0			Day 1			Day 2		
	Urine + wash		Faeces	Urine + wash		Faeces	Urine + wash		Faeces
Rat	Vol	pH	(gm)	Vol	pH	gm	Vol	pH	gm
1	35.0	n.c.	n.c.	20.0	n.c.	n.c.	29.0	n.c.	n.c.
2	15.0	n.c.	n.c.	15.0	n.c.	n.c.	14.0	n.c.	n.c.
5	13.0	6.5	11.18	14.0	6.7	6.68	21.0	7.0	8.04
6	11.0	6.7	9.03	8.0	6.4	7.32	10.0	7.0	7.66
7	15.0	7.0	8.8	19.5	7.0	5.67	9.5	7.0	7.04
8	18.0	7.0	8.99	11.0	6.7	4.42	18.0	7.0	8.24
Mean	18	6.8	10	15	6.7	6	17	7.0	8
sd	9	0.2	1	5	0.2	1	7	0.0	1

n.c. - not collected

B) Dose = 1.49 mmol/kg

	Day 0			Day 1			Day 2		
	Urine + wash		Faeces	Urine + wash		Faeces	Urine + wash		Faeces
Rat	Vol	pH	(gm)	Vol	pH	gm	Vol	pH	gm
3	15	6.4	n.c.	17	6.7	n.c.	15	7.0	5.38
4	20	6.7	n.c.	18	7.0	7.49	18	7.0	11.32
5	16	6.7	9.39	14	6.4	6.91	24	6.7	11.96
6	17	7.0	6.29	9	6.4	6.33	23	7.0	8.65
7	28	7.0	4.74	18	6.7	5.65	17	7.0	7.01
8	18	7.0	8.05	16	6.4	5.94	12	7.0	6.17
Mean	19	6.8	7	15	6.6	6	18	7.0	8
sd	5	0.2	2	3	0.2	1	5	0.1	3

n.c. - not collected

Appendix 3: Supporting data for *p*-cymene metabolism in the brushtail possum

A3.1. Introduction

The following tables support the data reported for *p*-cymene metabolism in brushtail possums presented in Chapter 4.

Table A3.1. Calibration curve concentrations and parameters for metabolites quantified in the brushtail possum.

		Cy 2	Cy 5	Cy 6	Cy 8
Stock soln conc (µmol/400µl methanol)		16.6	14.4	8.6	8.4
Dilution #	Stock soln[1] µl			µmol/ml	
1	0	0.00	0.00	0.00	0.00
2	10	0.42	0.36	0.22	0.21
3	20	0.83	0.72	0.43	0.42
4	30	1.25	1.08	0.65	0.63
5	40	1.67	1.44	0.86	0.84
6	50	2.08	1.80	1.08	1.06
n (calibration curves)		2	2	2	2
Equations (mean)		y=0.801x - 0.001	y= 1.081x - 0.078	y= 0.860x - 0.01	y=0.804x -0.003
Conc ranges (µmol/ml)		0.00 - 2.08	0.00 - 1.80	0.00 - 1.08	0.00 - 1.06
R^2 (mean)		0.998	1	0.999	1

[1]Stock solutions were diluted to 1 ml with blank urine.

Table A3.2. Individual brushtail possum details including sex, weight, dose and absolute molar recovery of each metabolite, excreted in 48 h, for each dose.

GG	Sex	Weight	Dose	Metabolite recovery (µmol)					
		kg	µmol	Cy 2	Cy 5	Cy 6	Cy 8	Cy 9	Total
Dose 0.37 mmol/kg									
Free									
1	f	2.12	790	39	275	75	44	99	532
2	m	4.62	1720	21	139	136	9	29	334
5	m	3.27	1220	39	194	116	13	28	390
6	f	2.8	1040	23	159	89	50	37	359
7	f	3.8	1420	22	209	60	39	57	386
8	f	2.33	867	28	143	69	82	43	365
Total									
1				48	394	119	9	116	687
2				10	533	191	33	110	877
5				15	354	159	82	60	669
6				50	490	131	16	132	820
7				31	418	89	35	102	675
8				24	206	80	66	55	431
Dose 1.49 mmol/kg									
Free									
3	f	2.12	3160	199	799	82	77	99	1255
4	m	4.10	6110	42	236	201	26	31	535
5	m	3.03	4510	166	629	250	103	173	1321
6	f	2.65	3950	159	670	267	64	124	1283
7	f	3.80	5660	161	569	223	19	28	999
8	f	2.26	3370	116	390	113	113	107	838
Total									
3				225	1269	154	262	133	2042
4				232	2956	212	220	457	4078
5				223	1452	196	519	387	2777
6				152	1062	171	249	175	1808
7				203	1538	158	356	162	2417
8				173	1188	103	963	248	2675

Table A3.3. Reconciling the urinary measurements of conjugated metabolites (total - free metabolite) with glucuronic acid (GA) after each dose of *p* -cymene in the brushtail possum.

	Day 0[1]		Day 1		Day 2	
BP	Conjugated metabolites	Urinary GA	Conjugated metabolites	Urinary GA	Conjugated metabolites	Urinary GA
			µmol/kg			
Dose 0.37 mmol/kg						
1	0	-	74	90	0	17
2	0	30	14	73	103	130
3	0	19	18	115	67	16
4	0	0	0	0	166	22
5	0	0	2	23	74	3
6	0	15	-	-	0	13
Mean	0	13	22	60	69	34
sd	0	13	30	47	64	48
P -value[2]				0.09		0.26
Dose 1.49 mmol/kg						
1	0	-	326	397	46	101
2	0	-	106	132	758	645
3	0	20	418	795	63	49
4	0	-	197	610	1	0
5	0	-	342	424	31	112
6	0	15	764	941	49	0
Mean	0	18	359	550	158	151
sd	0	4	228	293	295	247
P -value[2]				0.04		0.83

[1]Day 0 is the pre-dose control, which was collected for 8 h. Days 1 and 2 were 24 h collections.

[2]Student's paired *t* -test comparing conjugated metabolites with GA.

"-" no urine voided.

nb. 1.0 µmole glucuronic acid = 194 µg

Table A3.4. Urine and faeces output for individual brushtail possums during *p* -cymene dosing experiments.

a) Dose = 0.37 mmol/kg

	Day 0[1]			Day 1				Day 2			
	Urine + wash		Faeces	Urine	Urine + wash		Faeces	Urine	Urine + wash		Faeces
BP	ml	pH*	g	ml	ml	pH	g	ml	ml	pH	g
1	102	8	44.58	-	142	8.7	10.38	-	140	8.4	20.74
2	75	8.4	13.3	74	152	8.1	28.1	57	96	7.2	12.3
5	16	8.4	5.49	26	86	8.7	3.83	83	137	7.5	0.58
6	4	8.4	-	1	56	7.2	3.64	46	83	7.2	6.09
7	1	7.1	-	24	63	8.7	16.69	42	95	7.5	19.76
8	28	8.1	6.34	-	-	-	3.83	90	144	8.4	-
Mean	38	8.1	17	31	100	8.3	11	64	116	7.7	12
sd	41	0.5	18	31	45	0.7	10	22	27	0.6	9

a) Dose = 1.49 mmol/kg

	Day 0[1]			Day 1				Day 2			
	Urine + wash		Faeces	Urine	Urine + wash		Faeces	Urine	Urine + wash		Faeces
BP	ml	pH	(g)	ml	ml	pH	(g)	Vol (ml)	Vol (ml)	pH	(g)
1	-	-	-	-	113	8.5	8.08	50	166	8.4	8.08
2	-	-	-	53	92	7.2	5.56	87	131	7.2	5.87
5	43	8.4	0.78	110	155	8.1	4.47	170	202	8.7	9.51
6	-	-	-	120	157	8.4	20.54	128	180	8.1	4.15
7	-	-	-	54	108	8.4	1.16	140	195	8.4	13.08
8	13	7.2	-	139	184	8.1	3.59	149	214	8.4	8.8
Mean	28	7.8		95	135	8.1	7	121	181	8.2	8
sd	21	0.8		39	36	0.5	7	44	30	0.5	3

*"-" no urine or faeces excreted.

[1]Day 0 (pre-dose) samples were only collected for 8 h, and some possum failed to urinate in this time.

Appendix 4: Report on deaths of ringtail possums while in captivity

Two possums (RT-1 and 6) died while acclimatising to captivity and a third (RT-4) died within 24 h of being administered an oral dose of *p*-cymene by gavage. The first two possums were being fed a mixed diet of *E. pulchella* and artificial diet, while the third, was consuming artificial diet alone. The artificial diet was the same as that used successfully by researchers in other institutions. Reports on the deaths of each possum were submitted to the University of Tasmania Ethics Committee and the Parks and Wildlife Service. All reports, including autopsy reports have been compiled.

1) Ringtail # 1 (RT-1; captured 29/8/96, died 29/9/96) a female possum weighing 1234 g on capture and 1090 g at death. She had been in captivity for three and a half weeks before her death. At the time of capture, the possum was a little mangy around the tail area. For the first two weeks of her captivity she was fed *E pulchella, ad lib,* and had maintained her body weight. She was sharing an enclosure with another female (carrying a pouch young). The artificial diet was introduced in the third week. Eucalypt leaf was removed from her enclosure over several days, during which time she lost about 10 % of her body weight. RT-1 was placed in an enclosure of her own after she developed sores at the base of her tail and was re-offered *E. pulchella* to regain weight. The afternoon leaves were re-offered, RT-1 got up and ate for about half an hour before returning to sleep. The following two nights both leaf and a small amount of artificial diet were eaten. Faeces were excreted each night and no further weight was lost. On the afternoon of the third day (ie. the day before she died), the possum was observed to be very sleepy and when aroused seemed only interested in tucking up and going back to sleep.

She was found the following morning on the floor of the enclosure. She was unconscious and very cold. She was placed in a bag and rewarmed with body heat. She regained consciousness briefly before dying. At the time of death the sores on the base of her tail were weeping.

An autopsy was arranged by Dr Barrie Wells (Kingston Animal Hospital) at the Animal Health Laboratories, Department of Primary Industry, Mt Pleasant, Launceston. The report concluded that the cause of death was a severely impacted stomach. The report was discussed with Dr W. J. Foley (animal physiologist, James Cook University, Townsville) who commented that in the number of ringtail possums and greater gliders he has dissected (both sick and healthy animals), the stomach contents normally appear dry and hard with very little content in the intestine below the stomach. Thus, there is doubt about the actual cause of death.

2) Ringtail # 6, (RT-6; captured 26/9/96, died 5/10/96) a male possum weighing 968 g on capture and 903 g at death. He had been in captivity for 10 days when he died. RT-6 had been captured from the same nest as RT-1, but 3 weeks later. He also appeared quite mangy at the time of capture. He was kept in an enclosure by himself, but would regularly escape into adjacent enclosures and share a nest box with the two ringtails in that enclosure. Five days after capture, a distended cloaca was noticed. It

appeared to be tender and swollen. He was seen by Dr Cameron Bell (veterinarian, Kingston Animal Hospital) who diagnosed a large abscess on the cloaca, possibly resulting from a cat attack prior to capture. Subcutaneous penicillin was administered.

The possum was returned to its enclosure and monitored. On the second night, there was no evidence that the possum had eaten or emerged from his nest box. The abscess showed no sign of improving so he was returned to the veterinarian (Dr Barrie Wells). An attempt to lance the abscess was unsuccessful and it was decided not to administer any further antibiotics. He was returned to a smaller cage fitted with a nest box and water (7% dextrose), and both leaf and artificial diet offered. Faeces were passed on the following two nights and the possum appeared to be eating well so was returned to its large enclosure on Friday. The possum was found unconscious, supine and hypothermic the following morning and was euthenased.

Unfortunately, the possum's body was incinerated before an autopsy could be arranged. Cause of death was probably due to a combination of the abscess, the administration of penicillin and the confounding effect of stress in the animal.

3) Ringtail # 3, (RT-3; captured 12/9/96, died 25/10/96) was another female possum weighing 1120 g on capture and 1079 g at death. She had an unfurred pouch young which was euthenased after her death. She had been in captivity for six and a half weeks. When captured she had slightly runny eyes, but these improved over a couple of weeks. Otherwise she seemed in good condition. She shared a nest box with a young male each night. All the time she was in captivity she was offered Eucalypt leaf and artificial diet. A week before her death she was maintaining her body weight on a full artificial diet. She was maintained on this diet for 6 days before she died.

The day before she died, we decided that this possum and 2 others were sufficiently acclimatised to the artificial diet to commence the *p*-cymene dosing experiment. The possums were transferred to metabolism cages for 24 h prior to the dose being administered. The possums were provided with artificial diet and 7% glucose in water. RT-3 drank 200 ml of glucose-water overnight and ate very little of the diet (20 g). The following night water (with no glucose) and diet were provided and she ate about 100 g of diet. After the *p*-cymene was administered (200 mg/kg), she neither ate nor drank. She excreted about 60 ml of urine but no faeces.

The dose was contained in peanut oil and administered by gavage. The administration of the dose appeared to go well with the possum happy to accept some banana immediately after the dose. When last checked, an hour and a half after the dose, she seemed settled and was sleeping. She was found dead early the next morning, and her pouch young was euthenased.

We were particularly concerned about this possum's death as it occurred after the administration of *p*-cymene. The possibility of its death as a direct consequence of the *p*-cymene had to be eliminated. Initially we considered the death may have been caused by massive liver failure resulting from the *p*-cymene dose. However, analysis of excreted urine revealed no *p*-cymene metabolites. Hepatotoxicity would presumably have been metabolite-mediated, yet, the *p*-cymene dose had not been metabolised or eliminated at the time of death. Furthermore, the time course for hepatic necrosis would be expected to take longer than observed here.

Dr Barry Munday (Aquaculture, University of Tasmania) is an experienced wildlife pathologist and agreed to perform an autopsy on RT-3. His autopsy reports suggested enteritis and associated dehydration to be the cause of death. The excessive glucose-water consumption two nights prior to her death may have been a symptom or even the cause, given the total load of glucose ingested. Liver damage was noted, but little acute damage observed.

Careful consideration was given to how to continue the study. It was decided to modify the experimental regimen to allow sufficient results to make the capture of the animals worthwhile. It was decided to dose the remaining possums with the lower dose of *p*-cymene (0.37 mmol/kg) and collect urine and faeces as planned. Two other experiments, which were to involve dosing the possums with other terpenes, were discarded as returning healthy possums to their habitat as soon as possible became a priority.

The dosing experiment was completed successfully with the remaining three possums. All possums seemed in good health and were re-introduced to a leaf diet for 4 nights and their weight monitored before they were released at dusk on the fifth night.

Appendix 5: Supporting data for *p*-cymene metabolism in ringtail possums

A5.1. Introduction

The following tables of data support the results reported for *p*-cymene metabolism in ringtail possums reported in Chapter 5.

Table A5.1. Calibration curve concentrations and parameters for metabolites quantified in the ringtail possum.

		Cy 2	Cy 3	Cy 6
Stock soln conc (µmol/400µl methanol)		11.9	6.5	19.3
Dilution #	Stock soln[1] µl		µmol/ml	
1	0	0.00	0.00	0.00
2	10	0.30	0.16	0.48
3	20	0.60	0.33	0.96
4	30	0.87	0.49	1.45
5	40	1.20	0.65	1.93
6	50	1.50	0.81	2.41
n (calibration curves)		2	2	2
Equations (mean)		y=0.743x - 0.085	y= 0.499x - 0.035	y=0.806x - 0.156
Conc ranges (µmol/ml)		0.00 - 1.50	0.00 - 0.81	0.00 - 2.41
R^2 (mean)		0.985	0.989	0.98

[1]Stock solutions were diluted to 1 ml with urine.

Table A5.2. Individual ringtail possum details including sex, weight, *p* -cymene dose, and molar recovery of each metabolite, excreted in 48 h, for each dose.

RT	Sex	Weight	Dose	Metabolite recovery (µmol)			
		kg	µmol	Cy 2	Cy 5	Cy 6	Total
A) Dose 0.37 mmol/kg							
Free							
1	f	1.234	na				
2	m	0.849	317	63	55	73	191
3	f	1.079	na				
4	m	1.000	360	94	59	106	260
5	f	1.127	405	91	93	145	329
6	m	0.932	na				
Total							
1							
2				83	54	86	223
3							
4				112	61	125	299
5				94	80	147	321
6							
B) Dose 1.49 mmol/kg							
Free							
5	f	1.117	1664	1482	102	1455	3039
Total							
5				1482	115	1293	2890

Table A5.3. Measured urinary glucuronic acid (µmol/kg) excretion after 0.37 mmol/kg *p* -cymene by ringtail possums (n = 3).

Glider	Urinary glucuronic acid µmol/kg		
	Day 0	Day 1	Day 2
2	17	2	0
4	27	15	4
5	82	201	1

[2]There was no significant difference in glucuronic acid levels for pre- and post-dose urine (Anova (single factor) $df = 1$, $F = 0.84$, $P = 0.48$).

Table A5.4. Urine and faeces output for individual ringtail possums during *p* -cymene dosing experiments.

RT	Day 0			Day 1			Day 2		
	Urine + wash		Faeces	Urine + wash		Faeces	Urine + wash		Faeces
	Vol[2]	pH	(gm)	Vol[2]	pH	gm	Vol[2]	pH	gm
Dose 0.37 mmoll/kg									
2	63 (25)	8.7	9.64	59 (18)	9	8.1	71 (25)	8.4	4.6
4	88 (68)	7.8	4.47	55 (20)	6.4	2.66	84 (35)	7.2	5.87
5[1]	120 (98)	8.1	11.22	130 (97)	7.2	23.53	44 (14)	9	3.91
Dose 1.49 mmol/kg									
2[3]	11	9.3	7	87 (63)	8.7	6.06	70 (20)	8.4	8.32
3	79	7.8	4	115 (68)	7.5	0	Died		
5[1]	48	8.7	12	96 (58)	8.1	10.82	120 (80)	9	12

[1]RT-5, urine and faeces included that excreted by pouch young. Dose was calculated from mothers weight only.
[2]Volumes are for urine plus cage washings. Neat urine volumes are reported in parentheses.
[3]RT-2 urine collection was incomplete as an unknwon amount of urine was lost.

Table A5.5. Intake of artifical diet for each ringtail possum during both *p* - cymene dosing experiments.

	Food intake (g/kg)			Food intake (g/kg)		
	Dose 1			Dose 2		
Ringtail	Day 0	Day 1	Day 2	Day 0	Day 1	Day 2
2	157	70	64	83	17	52
3	-	-	-	90	died	-
4	62	68	96	-	-	-
5	107	216	92	121	203	163

Appendix 6: Supporting data for *p*-cymene metabolism in greater gliders

A6.1 Introduction

The following tables of data support the results reported for *p*-cymene metabolism in greater gliders in Chapter 6.

Table A6.1. Calibration curve concentrations and parameters for each metabolite quantified in the greater glider.

		Cy 2	Cy 5	Cy 6
Stock soln conc (µmol/400µl methanol)		23.8	27.1	21.5
Dilution #	Stock soln[1] µl		µmol/ml	
1	0	0.00	0.00	0.00
2	10	0.60	0.68	0.54
3	20	1.19	1.36	1.08
4	30	1.79	2.03	1.62
5	40	2.38	2.71	2.15
6	50	2.98	3.39	2.69
7	70	4.17	4.75	3.77
n (calibration curves)		2	2	2
Equations (mean)		y=0.476x + 0.011	y= 0.463x - 0.003	y=0.591x + 0.007
R^2 (mean)		0.972	0.955	0.958
Conc ranges (µmol/ml)		0.60 - 4.17	0.68 - 4.75	0.54 - 3.77

[1]Dilutions of stock solutions were added to 1 ml of diluted urine.

Table A6.2. Individual greater glider details including sex, weight, *p* -cymene dose, and molar recovery of each metabolite, excreted in 48 h.

GG	Sex	Weight	Dose	Metabolite recovery µmol			
		kg	µmol	Cy 2	Cy 5	Cy 6	Total
Dose 0.37 mmol/kg							
Free							
A	f	0.726	1081	122	386	408	916
B	f	0.62	923	78	234	269	581
D	m	0.594	885	76	219	333	628
E	f	0.584	870	96	283	268	647
F	f	0.762	1135	63	281	367	712
G	f	0.676	1007	114	293	377	785
Total							
				99	313	349	762
				95	291	289	675
				85	292	367	745
				86	293	249	628
				58	290	357	705
				95	323	369	787

Table A6.3. Average daily urinary glucuronic acid (µmol/kg) excreted by greater gliders (from post-dose measurements) and pre- and post-dose glucuronic acid, normalised by urinary creatinine concentration for comparison.

Glider	Glucuronic acid	Urinary GA/creatinine (µmol)[2]	
	µmol/kg/day[1]	pre-dose	post-dose[1]
A	541	-	2
B	327	12	5
D	1971	14	8
E	4605	14	39
F	5031	5	26
G	3089	2	19

[1]Glucuronic acid and glucuronic acid/creatinine ratios are the average of 2 days post-dose measurements.
[2]There was no significant difference between glucuronic acid/creatinine ratio for pre- and post-dose urine (Anova (single factor) $df = 1$, $F = 2.2$, $P = 0.17$; $n = 5$).
nb. Urinary glucuronic acid was normalised with urinary creatinine to allow a comparison between pre- and post doses levels, as pre-dose samples were not collected for a full 24 h.

Table A6.4. Urine and faeces output for individual greater gliders during *p* -cymene dosing experiments.

	Day 0			Day 1			Day 2		
	Urine + wash		Faeces	Urine + wash		Faeces	Urine + wash		Faeces
GG	Vol	pH	(gm)	Vol	pH	gm	Vol	pH	gm
Dose 1.49 mmol/kg									
A	3	5.2	4.79	62	5.8	5.58	82	6.1	19.34
B	9	5.2	2.94	31	6.1	2.3	-	-	-
D	5	5.2	1.66	63	5.5	10.7	60	5.8	17.19
E	11	5.5	0.12	102	5.8	11.04	91	5.5	12.89
F	5	5.5	1.53	100	5.5	5.2	87	5.5	13.78
G	5	5.5	3.29	62	5.5	8.42	79	5.5	17.2

nb: urine was frozen immediately, thus neat urine volumes could not be measured.

Table A6.5. Weights (g) of individual greater gliders throughout their captivity.

Date	Greater glider weight (g)					
	A	B	D	E	F	G
13-Jun-99	788.0	676.0	622.0	604.0	800.0	736.0
18-Jun-99	751.0	639.0	608.0	599.0	742.0	704.0
20-Jun-99	-	-	594.0	584.0	762.0	-
22-Jun-99	726.0	620.0	-	-	-	676.0
24-Jun-99	723.5	677.0	-	-	-	-
25-Jun-99	-	619.0	611.0	603.0	791.0	-
27-Jun-99	736.0	562.0	586.0	575.5	794.5	687.0
Weight loss g	52	114	36	29	6	49
%	6.6	16.9	5.8	4.7	0.7	6.7

nb. wieght losses up to 10 % of captured weight were considered acceptable.

Appendix 7: Supporting data for *p*-cymene metabolism in koalas

A7.1. Introduction

The following tables support the data for *p*-cymene metabolism in koalas presented in Chapter 7.

Table A7.1. Calibration curve concentrations and parameters for metabolites quantified in the koala.

		Cy 2	Cy 5[2]	Cy 6	Cy 10
Stock soln conc[1] (µmol/400µl)		11.13	18.87	40.09	13.89
Dilution #	Stock soln µl			µmol/ml	
1	0	0.00	0.00	0.00	0.00
2	10	0.28	0.47	1.00	0.33
3	20	0.56	0.94	2.01	0.65
4	30	0.84	1.42	3.01	0.98
5	40	1.11	1.89	4.01	1.31
6	50	1.39	2.36	5.01	1.63
n (calibration curves)		3	2	3	3
Equations (mean)[3]		y=0.950x + 0.061	y=0.642x + 0.175	y=1.707x + 0.581	y=1.250x + 0.104
Metabolite/IS range		0.04 - 2.66	0.17 - 3.43	0.30 - 17.30	0.09 - 4.16
R^2 (mean)		0.99	0.99	0.99	0.99
CoV % (slopes)		4.6	0.8	2	0.5

[1]Dilutions of stock solutions were added to 1 ml of diluted urine.

[2]Cy 5 was doubly derivatised and the calibration curve reported here is for that derivative

[3]Significant y-intercept values are due to background *p* -cymene being present in the leaf.

Table A7.2. Individual koala details including sex, weight, dose and molar recovery of each metabolite, excreted in 48 h, for each dose.

K	Sex	Weight kg	Dose µmol	Metabolite recovery µmol Cy 2	Cy 6	Cy 10	Cy 5	Cy 13
A) Dose 0.37 mmol/kg								
Unhydrolysed								
1	M	9.94	495	542	766	144	157	1749
2	M	8.59	430	182	479	88	174	1263
3	M	10.09	505	156	933	59	267	1650
4	M	8.14	407	233	577	329	427	1016
5	M	9.24	462	913	1789	651	940	3098
6	M	7.14	357	78	597	212	396	1003
Hydrolysed								
1				361	408	177	384	1974
2				188	568	168	198	969
3				168	807	83	188	1670
4				229	595	307	73	1009
5				1050	2092	652	607	2874
6				88	703	196	81	1071
B) Dose 1.49 mmol/kg								
Unhydrolysed								
1		9.89	15067	1004	3362	1142	789	7085
2		8.34	12694	755	2504	179	956	5141
3		9.74	14799	1214	3090	441	1121	6461
4		8.05	12530	807	1337	797	1086	4395
5		9.30	14045	992	3312	1635	287	6500
6		7.40	11746	421	2092	668	464	4149
Hydrolysed								
1				791	2277	841	590	7606
2				709	2250	255	968	5076
3				1054	2596	517	999	6111
4				852	1652	945	1723	4979
5				1112	3353	1955	315	8257
6				435	2058	764	754	6487

Table A7.3. Urine and faeces output for individual koalas during *p* -cymene dosing experiments.

A) Dose 0.37 mmol/kg

Koala	Day 0				Day 1				Day 2			
	Urine (ml)			Faeces	Urine (ml)			Faeces	Urine (ml)			Faeces
	Urine	Wash	pH	g	Urine	Wash	pH	g	Urine	Wash	pH	g
1	160	95	5.8		-	-	-		171	100	5.8	
2	140	116	5.8		190	94	5.5		72	100	6.1	
3	78	118	5.8		65	94	5.5		97	99	5.5	
4	-	90	-		220	-	5.8		155	90	6.1	
5	30	93	5.8		96	73	6.1		110	80	5.5	
6	58	98	5.5		167	255	5.5		94	88	5.5	

B) Dose 1.49 mmol/kg

Koala	Day 0				Day 1				Day 2			
	Urine (ml)			Faeces	Urine (ml)			Faeces	Urine (ml)			Faeces
	Urine	Wash	pH	g	Urine	Wash	pH	g	Urine	Wash	pH	g
1	68	104	5.8		-	-	-		102	96	5.8	
2	68	95	5.5		98	94	5.8		78	93	5.2	
3	66	94	5.8		75	94	5.8		53	104	5.5	
4	63	95	5.8		162	87	5.8		102	98	5.5	
5	134	104	5.5		26	92	5.5		176	86	5.2	
6	152	83	5.2		130	85	4.9		97	77	5.2	

Appendix 8: Supporting data for 1,8-cineole metabolism in brushtail possums

A8.1. Introduction

The figures and tables in this appendix support the data reported for 1,8-cineole metabolism in brushtail possums presented in Chapter 10. Figures and Tables have been referred to in the text.

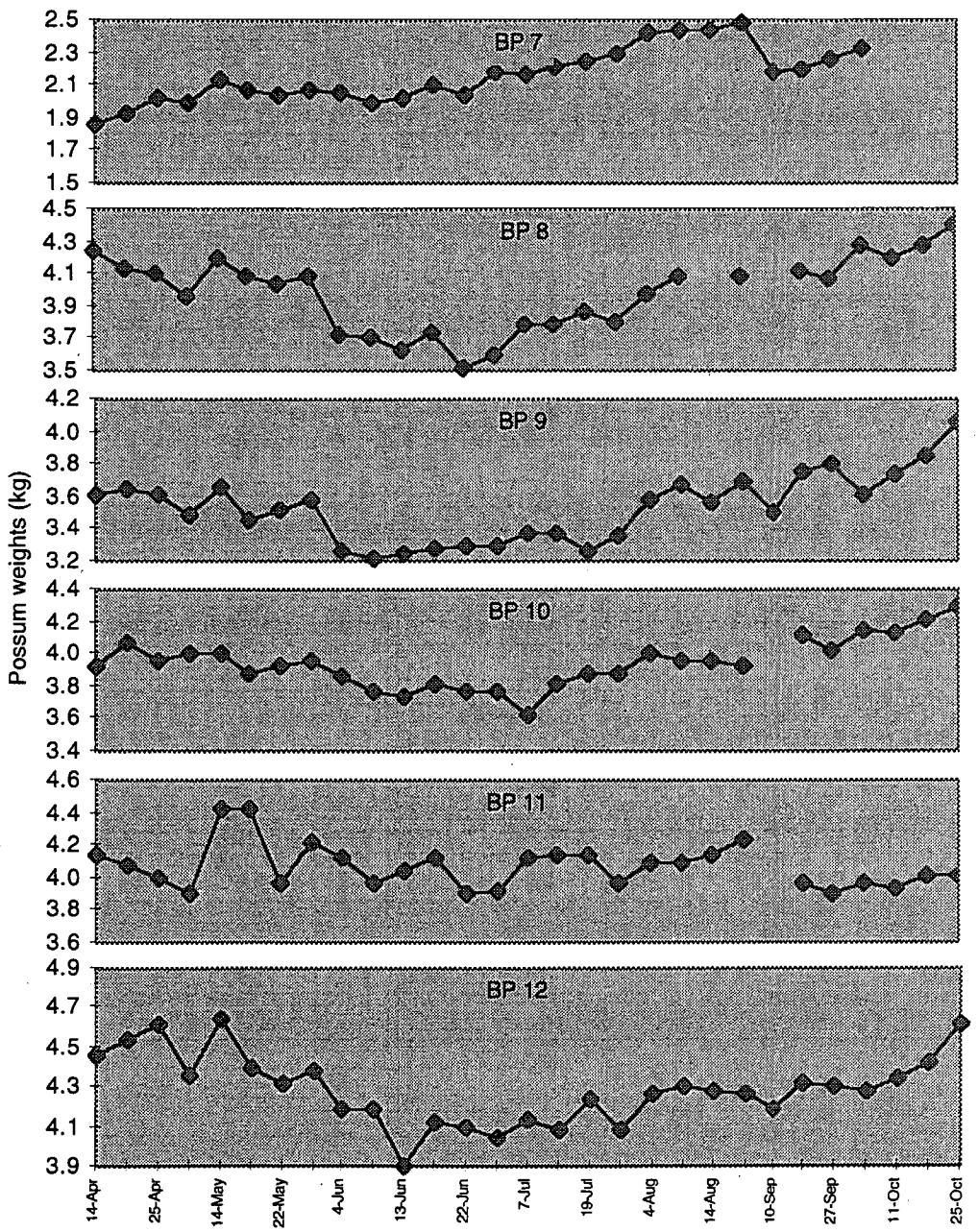


Figure A8.1. Brushtail possums weights (kg) were monitored regularly and are reported for each possum for the duration of their captivity.

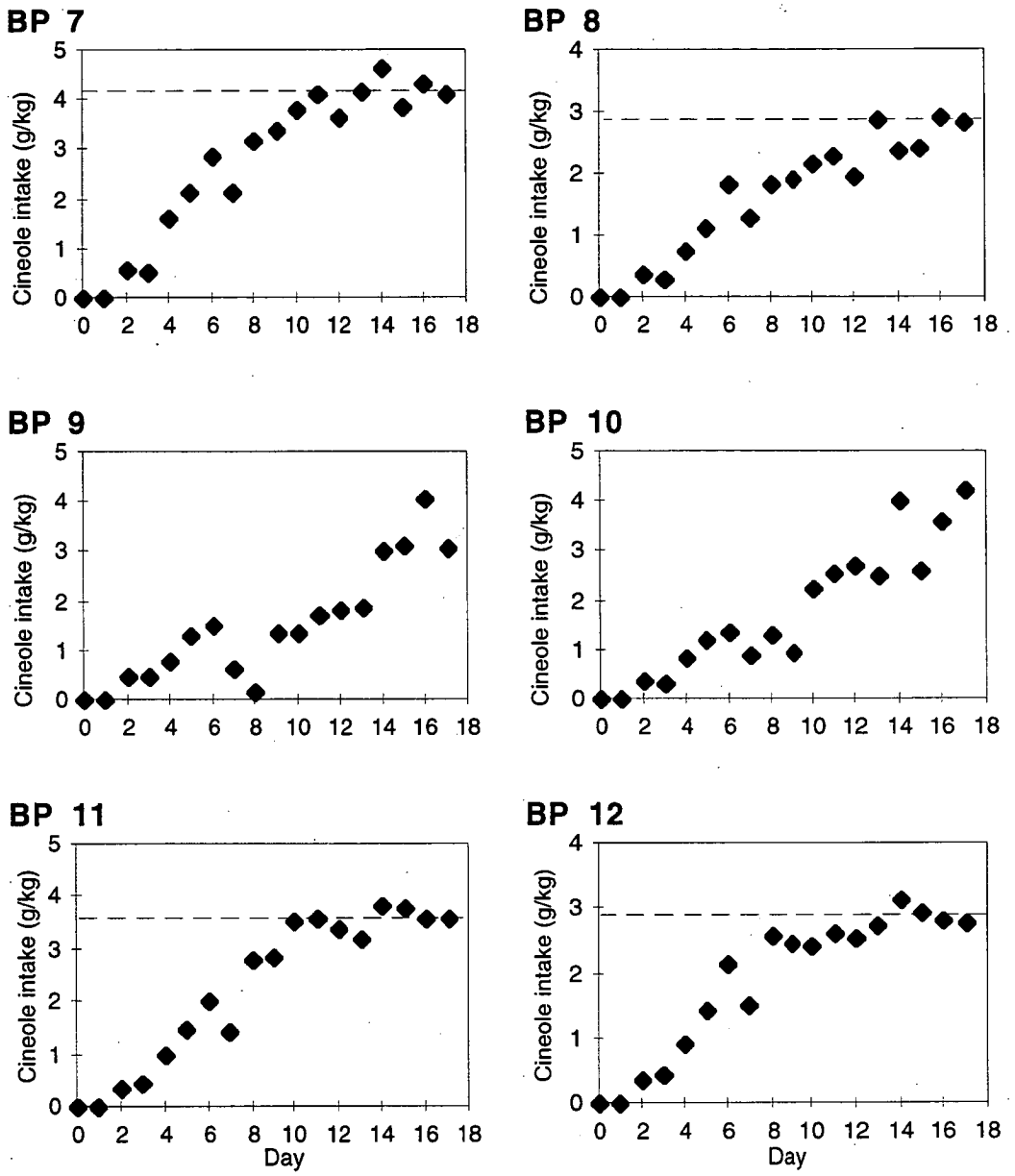


Figure A8.2. Daily 1,8-cineole intake (g/kg) for individual possums in feeding Experiment 1. Dashed lines indicate the approximate intake threshold for individual possums. Intakes for BP 9 and 10 were variable and therefore inconclusive.

Table A8.1. Individual brushtail possum details including sex, weight, 1,8-cineole intake and molar recovery for each metabolite in Experiment 1.

	Sex	Weight	Cineole	Metabolite recovery µmol								
Possum	Weight	kg	mmol	Ci 1	Ci 2	Ci 3	Ci 4	Ci 5	Ci 6	Ci 7	Ci 8	Ci 9
Day 3												
Free												
7	f	2.00	11.6	0	623	118	41	94	83	68	133	34
8	m	3.96	12.5	128	995	398	80	147	319	195	321	336
9	m	3.48	17.1	0	1171	287	104	0	265	204	469	345
10	f	4.00	14.4	0	368	0	101	0	322	243	235	262
11	m	3.90	18.4	0	1028	303	70	90	204	158	301	201
12	m	4.36	20.5	96	2501	1230	168	270	361	313	502	403
Total												
7				44	821	239	39	90	112	81	68	125
8				253	1441	637	116	146	550	368	853	827
9				108	2092	1098	98	174	287	266	361	377
10				145	1652	0	89	130	2881	305	283	348
11				87	1289	707	94	127	293	307	1052	646
12				169	3081	1458	153	341	363	283	482	395
Day 9												
Free												
7			32.4	0	3679	982	455	268	702	408	565	1207
8			36.0	254	3141	1811	515	597	1427	689	520	1201
9			23.0	0	725	158	67	51	211	175	229	1931
10			18.2	0	1013	131	109	67	468	297	206	188
11			52.4	217	4074	1728	549	383	1577	1046	1179	1656
12			50.6	40	1067	62	347	0	1835	1091	1077	1205
Total												
7				679	5178	3571	449	878	1385	801	573	1885
8				1015	4333	3267	474	916	2580	1226	652	2482
9				41	953	349	56	90	3615	83	69	61
10				65	1285	266	109	70	1167	366	374	357
11				603	6806	4602	539	934	1742	1082	749	1294
12				637	7356	3214	715	688	3419	2067	1823	2627
Day 17												
Free												
7			53.5	0	1760	425	410	231	1131	534	543	1708
8			73.0	111	3618	1050	895	48	2681	1278	831	1590
9			68.8	0	1528	204	379	0	2215	1262	756	1275
10			109.6	6	1537	0	493	0	3132	1443	763	2485
11			90.1	16	301	0	107	0	1328	792	720	605
12			79.0	14	629	51	288	0	2627	1647	770	1060
Total												
7				698	8791	5332	1017	1215	2281	1004	1351	3047
8				281	7117	3663	1043	806	2484	1139	344	908
9				441	5924	2342	596	484	362	1929	2078	0
10				74	10126	4968	1413	867	5644	2418	1645	4233
11				173	4491	0	483	275	2393	1308	772	919
12				487	7389	3284	910	519	3940	2246	1389	2084

1 of 2. Continued over page.....

Table A8.1. continued.

Possum	Ci 10	Ci 11	Ci 12	Ci 13	Ci 15	Ci 17	Ci 18	Ci 19	Ci 20	Total
Day 3										
Free										
7	42	27	107	46	75	79	0	539	0	2109
8	52	63	254	53	153	97	75	866	151	4683
9	92	91	329	83	213	242	56	2025	228	6203
10	35	37	367	59	96	152	21	1379	278	3954
11	60	44	197	59	125	104	54	903	103	4005
12	30	99	374	131	237	294	182	2214	0	9406
Total										
7	24	23	109	15	33	65	30	599	0	2516
8	90	211	404	69	654	108	91	458	0	7275
9	41	96	297	64	277	176	93	1515	0	7418
10	48	74	470	47	155	134	69	1154	0	7982
11	197	243	362	0	623	123	70	93	0	6314
12	72	97	367	98	295	269	123	2639	0	10683
Day 9										
Free										
7	117	308	841	200	668	537	0	5156	917	17012
8	100	170	1106	95	329	392	170	4041	1418	17976
9	50	52	216	49	130	103	36	606	99	4886
10	39	22	360	44	44	96	0	724	185	3994
11	245	291	1610	194	750	690	195	7977	974	25333
12	229	136	1841	0	332	666	0	4557	874	15359
Total										
7	105	343	1480	127	613	398	288	4130	0	22883
8	88	326	2024	0	521	337	258	3534	3019	27051
9	90	16	107	28	58	77	33	571	158	6456
10	59	43	501	0	105	87	0	422	0	5275
11	140	275	1695	209	606	514	307	7420	629	30146
12	323	387	3251	193	989	744	254	5475	0	34161
Day 17										
Free										
7	130	326	1136	156	594	621	178	4717	1428	16028
8	145	183	1985	0	350	679	0	6826	3234	25503
9	154	142	2186	115	356	573	0	5671	1112	17927
10	139	280	2375	0	532	782	0	6075	2422	22464
11	152	68	1018	99	159	325	0	370	654	6715
12	185	92	2503	0	0	693	0	4950	1045	16554
Total										
7	395	648	1777	186	1615	606	406	4643	1116	36129
8	57	107	1656	137	0	568	344	6882	0	27536
9	315	1027	0	537	393	543	0	4861	0	21832
10	223	633	4154	0	1181	748	359	6073	0	44759
11	159	104	1788	132	292	349	108	4985	0	18732
12	225	255	3360	0	642	700	212	5733	0	33374

2 of 2.

Table A8.2. Reconciling the urinary measurements of conjugated metabolites (total - free) with glucuronic acid in possums fed a diet containing increasing concentrations of 1,8-cineole in Experiment 1.

	Day 3 (0.5 % 1,8-cineole)		Day 9 (2.0 % 1,8-cineole)		Day 17 (4.0 % 1,8-cineole)	
Possum	Conjugated metabolites	Urinary GA	Conjugated metabolites	Urinary GA	Conjugated metabolites	Urinary GA
	µmol/kg					
7	204	129	2936	3438	10050	5268
8	655	90	2291	1567	514	3149
9	348	643	450	265	1119	2219
10	1007	562	320	187	5574	3623
11	592	216	1234	2226	3081	1611
12	293	1032	4312	2730	3858	2650
Mean	517	445	1924	1736	4033	3087
se	121	150	635	539	1419	522
p-value		0.74		0.63		0.41

Students *t* -test comparing conjugated metabolites with measured glucuronic acid.
nb. 1.0 µmol glucuronic acid = 194 g.

Table A8.3. Individual brushtail possum details including sex, weight, 1,8 cineole intake and molar recovery for each metabolite in Experiment 2.

	Sex	Weight	Cineole	Metabolite recovery µmol							
Possum	Weight	kg	mmol	Ci 1	Ci 2	Ci 3	Ci 4	Ci 5	Ci 6	Ci 7	Ci 8
Day 3											
Free											
7	f	2.02	29.6	13	768	1791	66	282	0	0	0
8	m	3.62	53.0	0	279	220	19	15	0	0	0
9	m	3.24	64.2	0	270	369	24	40	0	0	0
10	f	3.73	59.2	0	580	116	74	0	0	0	0
11	m	4.05	98.4	0	486	250	43	57	39	15	7
12	m	3.90	37.7	0	986	1109	55	156	69	44	97
Total											
7				245	936	1969	64	277	92	73	168
8				83	370	632	25	107	65	28	55
9				65	336	525	27	79	54	0	50
10				386	1290	2074	87	366	269	128	185
11				154	939	1437	54	207	162	96	404
12				147	1610	2045	93	167	212	164	413
Day 4											
Free											
7			39.0	19	1434	495	151	0	123	107	293
8			50.1	0	999	274	158	228	247	139	205
9			65.7	35	1751	1008	108	161	209	138	184
10			81.6	13	659	174	120	0	299	176	199
11			102.3	78	2897	1609	196	320	499	315	303
12			56.4	0	1042	256	100	0	182	153	168
Total											
7				342	3838	4010	218	654	434	334	583
8				592	2974	3411	271	550	1012	518	514
9				168	1983	1337	123	210	532	394	361
10				0	2782	0	285	0	0	0	0
11				460	3783	3248	211	648	1391	921	793
12				177	2203	1516	136	241	508	368	585
Day 5											
Free											
7			46.5	17	381	97	91	0	356	265	438
8			62.1	8	433	88	98	0	364	216	177
9			73.5	32	1778	693	113	122	321	217	254
10			51.4	0	116	10	18	0	142	81	80
11			101.3	0	456	55	103	0	679	415	835
12			60.3	0	605	103	79	416	485	320	860
Total											
7				385	4498	2729	378	507	1160	1115	4623
8				283	1946	1366	200	246	1230	647	401
9				180	1997	1235	124	198	780	487	457
10				80	1264	369	120	89	870	489	249
11				252	4322	2316	365	427	1715	1082	881
12				226	3964	2402	334	263	1118	717	1966

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Table A8.3. continued.

Possum	Ci 9	Ci 10	Ci 11	Ci 12	Ci 13	Ci 15	Ci 17	Ci 18	Ci 19	Ci 20	Total
Day 3											
Free											
7	27	0	0	14	56	57	0	78	707	103	3971
8	0	0	0	0	0	0	12	0	0	0	554
9	0	0	0	0	0	0	23	25	138	27	923
10	0	0	0	0	0	0	66	0	635	0	1481
11	18	0	12	25	25	20	64	15	659	101	1847
12	0	0	19	40	20	68	86	24	1007	0	3793
Total											
7	259	25	106	86	45	262	42	72	308	0	5038
8	70	7	28	29	6	61	14	15	101	0	1702
9	37	4	23	33	0	52	20	17	65	0	1394
10	351	0	101	216	0	195	53	85	379	0	6176
11	87	78	144	57	0	199	29	60	133	0	4250
12	119	68	83	144	0	227	50	66	20	0	5641
Day 4											
Free											
7	358	52	129	167	108	352	368	74	3924	145	8306
8	177	31	39	198	46	103	213	22	2291	313	5693
9	186	30	38	185	88	54	238	100	2874	348	7743
10	248	23	34	266	47	41	206	23	1943	390	4872
11	293	56	44	450	140	118	282	91	4474	553	12729
12	56	28	17	196	54	50	201	14	1732	76	4337
Total											
7	925	113	286	601	69	678	332	337	2581	0	16343
8	1048	79	210	795	0	413	202	264	1872	0	14734
9	549	80	93	580	63	230	211	112	1938	0	8968
10	0	0	0	0	0	0	0	0	428	0	3505
11	1149	158	220	1270	91	467	289	275	3190	0	18575
12	587	96	106	508	51	269	190	128	1082	0	8764
Day 5											
Free											
7	253	92	125	442	103	325	467	60	4145	238	7902
8	225	32	24	245	0	77	174	18	1640	304	4133
9	195	52	48	275	86	124	268	51	2998	386	8023
10	64	2	5	102	0	21	41	0	241	42	977
11	635	145	85	503	0	288	291	0	2921	0	7421
12	517	129	99	345	0	317	189	0	752	0	5228
Total											
7	3719	911	1882	1292	0	4388	508	339	0	0	28442
8	737	79	103	833	0	186	160	140	1346	0	9911
9	830	107	114	712	61	299	222	117	1788	0	9718
10	348	36	40	685	0	101	104	46	644	0	5545
11	1037	207	156	1498	164	457	360	227	4570	0	20047
12	1212	326	272	600	0	709	147	0	65	0	14334

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Table A8.3. continued.

Possum	Sex Weight	Weight kg	Cineole mmol	Metabolite recovery µmol Ci 1	Ci 2	Ci 3	Ci 4	Ci 5	Ci 6	Ci 7	Ci 8
Day 7											
Free											
7			56.9	99	1341	257	277	0	2486	1268	1514
8			62.9	24	460	73	170	0	2152	1074	829
9			64.4	37	1982	394	385	0	2922	1888	922
10			83.6	22	794	153	265	0	2359	1248	642
11			94.8	23	558	75	208	0	2063	1318	857
12			63.6	48	1990	479	292	0	1279	891	762
Total											
7				180	13313	7360	1379	1761	1943	884	618
8				576	4925	2818	605	383	3414	1568	4036
9				517	7238	2927	827	645	4513	2657	1265
10				661	7026	3400	870	699	3751	1692	2843
11				483	8145	3615	877	752	4947	2825	4235
12				759	7909	3718	678	706	3431	2142	1213
Day 8 - first washout day											
Total											
7				178	2371	987	211	266	650	350	380
8				142	2319	1025	258	317	651	343	184
9				55	1957	511	157	103	1008	634	169
10				134	4052	1348	412	372	1559	905	342
11				180	6220	2255	455	483	1905	1241	352
12				44	1733	495	145	94	402	243	153
Day 9 - second washout day											
Total											
7				0	71	18	0	0	0	0	0
8				0	147	16	13	0	0	0	0
9				0	265	56	20	0	0	0	0
10				0	228	43	18	0	70	52	0
11				0	482	83	37	0	147	127	65
12				0	297	78	0	0	0	0	0

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Table A8.3. continued.

Possum	Ci 9	Ci 10	Ci 11	Ci 12	Ci 13	Ci 15	Ci 17	Ci 18	Ci 19	Ci 20	Total
Day 7											
Free											
7	3025	275	509	1862	172	1089	952	0	6485	633	22253
8	1242	143	129	1463	0	353	332	0	2406	0	10856
9	1710	196	175	2990	180	43	841	0	7106	772	22550
10	1487	115	152	1876	0	312	628	0	4377	868	15308
11	885	200	87	1711	0	220	526	0	5504	589	14837
12	716	134	79	1105	110	215	528	58	3380	440	12519
Total											
7	1613	125	226	1562	303	520	1301	1003	453	0	34549
8	2078	736	1065	1752	0	2391	189	0	0	0	26545
9	3159	283	324	4198	0	673	748	311	5174	0	35469
10	3843	471	757	2242	0	1377	233	0	169	0	30044
11	3523	933	580	352	0	1605	553	328	0	0	33764
12	2029	209	251	2882	222	525	695	364	3710	0	31455
Day 8 - first washout day											
Total											
7	687	97	150	458	37	278	187	131	2191	419	10026
8	338	45	67	379	0	105	158	124	2497	943	9894
9	41	55	45	708	66	0	139	50	1869	290	7857
10	574	62	88	1207	85	132	236	142	4947	547	17145
11	366	0	134	764	379	121	346	517	17937	4259	37913
12	120	0	22	210	50	0	96	33	1122	183	5144
Day 9 - second washout day											
Total	0	0	0	0	0	0	0	0	0	0	89
7	0	0	0	0	0	0	5	0	0	0	182
8	0	0	0	0	0	0	12	0	0	0	353
9	0	0	0	0	0	0	0	0	0	0	412
10	59	0	0	86	0	0	0	0	0	0	1085
11	0	0	0	0	0	0	0	0	0	0	375

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Table A8.4. Reconciling the urinary measurements of conjugated metabolites (total - free) with glucuronic acid in possums challenged with a diet containing 4 % 1,8-cineole in Experiment 2.

	Day 3		Day 4		Day 5		Day 7	
Possum	Conjugated metabolites	Urinary GA	Conjugated metabolites	Urinary GA	Conjugated metabolites	Urinary GA	Conjugated metabolites	Urinary GA
	µmol/kg							
7	528	195	3979	4111	10168	3044	6087	8372
8	317	312	2497	2291	1596	1556	4334	2843
9	145	114	378	344	523	712	3987	2929
10	1259	1002	366	1517	1225	520	3950	2629
11	593	574	1444	474	3117	2442	4673	2878
12	474	139	1135	1005	2335	1050	4855	1999
Mean	553	389	1633	1624	3161	1554	4648	3608
se	156	140	569	577	1448	410	323	963
p-value		0.06		0.97		0.21		0.20

Students t-test comparing conjugated metabolites with measured glucuronic acid.

Table A8.5. Individual brushtail possum details including sex, weight, 1,8-cineole intake and molar recovery for each metabolite in Experiment 3.

	Sex	Weight	Cineole	Metabolite recovery µmol								
Possum	Weight	kg	mmol	Ci 1	Ci 2	Ci 3	Ci 4	Ci 5	Ci 6	Ci 7	Ci 8	Ci 9
Day 3												
Free												
7	f	2.44	28.6	40	2089	1058	98	57	126	150	1152	628
8	m	4.08	47.8	286	1822	1851	95	310	288	184	331	474
9	m	3.57	32.7	0	355	117	18	0	43	43	86	14
10	f	3.95	36.6	257	1480	1394	119	180	156	104	653	603
11	m	4.14	44.9	0	4	3	0	0	0	0	0	0
12	m	4.28	54.5	0	2305	804	85	0	336	545	1280	546
Total												
7				44	2297	1164	108	0	139	165	1267	691
8				421	2157	3094	127	558	239	160	264	414
9				18	423	293	24	34	0	0	0	0
10				554	1563	2714	0	0	155	115	556	605
11				0	9	16	0	0	0	0	0	0
12				139	2776	1483	147	247	447	568	2831	1528
Day 4												
Free												
7			34.0	0	1355	261	117	0	371	282	1324	891
8			51.7	98	2268	724	265	191	1488	852	633	979
9			40.3	15	2686	329	185	0	624	486	726	761
10			61.8	35	2230	260	389	0	2285	1489	2214	169
11			64.9	158	2687	1003	143	241	953	632	3189	1626
12			56.6	26	1536	298	150	0	594	559	500	468
Total												
7				196	3265	1913	201	460	503	468	636	548
8				706	444	3533	389	839	1953	981	3371	2718
9				213	3757	2217	204	335	675	506	3041	1934
10				774	6921	4079	676	888	2987	1461	1469	2797
11				603	4188	3390	243	618	1235	799	3754	940
12				168	3210	1356	219	223	769	661	635	471
Day 6												
Free												
7			41.8	0	1552	267	162	98	933	715	1685	1029
8			50.1	156	2812	863	342	263	2821	1566	1638	2025
9			48.6	0	738	68	212	0	2672	1814	1659	2402
10			32.7	21	693	72	221	0	3776	1885	877	2712
11			65.2	0	198	0	64	0	817	519	483	432
12			48.3	0	2113	320	259	95	1404	969	2007	1195
Total												
7				358	3355	1907	266	491	1039	710	2692	842
8				656	4764	2907	468	802	3117	1563	944	2339
9				442	7137	3133	651	538	2951	1937	1146	2332
10				513	7876	3088	807	627	3878	1717	919	2164
11				173	2322	935	194	231	1092	634	532	793
12				0	3616	1224	289	251	748	455	299	148

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Table A8.5. continued.

Possum	Ci 10	Ci 11	Ci 12	Ci 13	Ci 15	Ci 17	Ci 18	Ci 19	Ci 20	Total
Day 3										
Free										
7	199	437	165	0	1008	120	0	0	0	7334
8	29	145	282	42	284	137	120	801	0	7489
9	0	12	45	0	26	0	0	59	0	828
10	85	381	157	0	621	39	0	0	0	6239
11	0	0	0	0	0	0	0	0	0	18
12	314	165	558	0	411	259	0	0	0	7620
Total										
7	218	481	181	0	1109	133	0	0	0	8004
8	39	118	225	34	303	117	158	803	0	9238
9	0	0	0	0	0	29	0	0	0	829
10	90	523	152	0	878	0	117	0	0	8033
11	0	0	0	0	0	0	0	0	0	36
12	578	4189	504	0	1515	264	0	0	0	17228
Day 9										
Free										
7	269	206	447	0	527	269	0	889	0	7216
8	98	116	1270	0	310	302	0	2652	0	12253
9	172	129	817	81	390	429	0	3336	0	11174
10	377	604	2136	0	993	453	0	0	0	13644
11	673	478	976	0	1005	227	0	0	0	14001
12	128	35	802	68	77	275	0	1432	0	6959
Total										
7	120	148	543	98	433	340	217	1952	0	12049
8	621	1062	1406	0	2264	331	314	587	0	21527
9	709	827	641	0	2250	365	0	356	0	18038
10	265	483	2365	0	849	546	348	3331	0	30249
11	686	884	906	0	2168	164	0	0	0	20590
12	151	91	883	140	119	311	137	1888	0	11444
Day 17										
Free										
7	267	421	1106	0	846	225	0	0	0	9313
8	332	355	2224	0	834	314	0	0	0	16553
9	356	269	3090	0	719	679	0	4145	0	18833
10	162	205	3274	0	413	549	0	3226	0	18095
11	84	39	737	0	134	152	0	910	0	4579
12	390	89	1094	0	3333	148	0	0	0	13428
Total										
7	484	823	929	0	1927	256	0	0	0	16085
8	178	301	2178	0	536	340	259	2166	0	23527
9	240	246	2878	0	671	685	336	5892	0	31227
10	86	279	2753	0	688	490	261	3387	0	29543
11	114	106	878	0	270	155	83	1010	0	9533
12	67	60	628	70	0	323	71	1520	0	9780

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Table A8.6. Reconciling the urinary measurements of conjugated metabolites (total - free) with glucuronic acid in possums pre-treated with probenecid and challenged with a diet containing 4 % 1,8-cineole in Experiment 3.

	Day 3		Day 4		Day 6	
Possum	Conjugated metabolites	Urinary GA	Conjugated metabolites	Urinary GA	Conjugated metabolites	Urinary GA
	µmol/kg					
7	275	1028	1981	1901	2776	2351
8	429	573	2273	2261	1709	1951
9	0	207	1922	1723	3471	6023
10	454	594	4204	5319	2898	5318
11	4	4	1592	1261	1197	1065
12	2245	334	1048	903	1484	1286
Mean	568	457	2170	2228	2256	2999
se	345	146	441	648	373	870
p-value		0.78		0.80		0.24

Students *t* -test comparing conjugated metabolites with measured glucuronic acid.

Appendix 9: Supporting data for 1,8-cineole metabolism in koalas

A9.1. Introduction

Results reported in Chapter 11 were derived from the data reported in the following table.

Table A9.1. Individual koala details including sex, weight, 1,8-cineole intake and molar recovery for each 1,8-cineole metabolite, excreted in 24 h after feeding on *E. cephalocarpa*.

Koala	Sex	Weight	Dose	Metabolite recovery µmol							
		kg	mmol	Ci 2	Ci 3	Ci 4	Ci 5	Ci 17	Ci 13	Ci 22	SUM
Free											
1	m	9.89	19.8	37	18	0	0	2273	19751	1512	23591
2	m	8.34	10.7	58	27	14	0	2042	17983	1829	21953
3	m	9.74	10.9	65	25	37	0	1008	9467	1096	11698
4	m	8.05	32.8	447	44	128	0	2574	27449	1454	32095
5	m	9.30	26.3	635	643	94	89	3652	33943	3613	42668
6	m	7.40	22.4	128	46	41	0	1986	18095	2784	23080
Total											
1			19.8	963	1044	106	176	2157	20911	1635	26991
2			10.7	246	315	40	35	1859	16508	1871	20874
3			10.9	295	315	573	0	1269	11668	1490	15610
4			32.8	859	463	210	73	2178	21990	1700	27472
5			26.3	1088	1529	161	278	3956	33015	3825	43851
6			22.4	368	196	76	0	1983	16860	2953	22436

Appendix 10: List of publications

Boyle, R., McLean, S., Foley, W.J. and Davies, N.W. (1999). Comparative metabolism of dietary terpene, *p*-cymene, in generalist and specialist folivorous marsupials. *Journal of Chemical Ecology*, 26 (9): 2109-2126.

Boyle, R., McLean, S., Foley, W.J., Moore, B.D., Davies, N.W. and Brandon, S. (In review). Fate of the dietary terpene, *p*-cymene, in the koala. *Journal of Chemical Ecology.*

Peterson, G.M., Boyle, R.R., Francis, H.W., Oliver, N.W.J., Paterson, J., Witt, R.J.v. and Taylor, G.R. (1990). Dosage prescribing and plasma oxipurinol levels in patients receiving allopurinol therapy. *European Journal of Clinical Pharmacology*, 39 : 419-421.